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TOXICOLOGICAL SCIENCES 134(2), 271–275 2013
doi:10.1093/toxsci/kft120
Advance Access publication June 6, 2013

Urothelial Cell Intracytoplasmic Inclusions After Treatment of Promyelocytic Leukemia With Arsenic Trioxide

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Received April 9, 2013; accepted May 15, 2013

Intramitochondrial inclusions containing arsenite that occur within urothelial cells have been previously described in mice exposed to high concentrations of arsenic but not in rats. In epidemiology studies, similar urothelial cell inclusions have also been observed in the urine of humans exposed to high concentrations of arsenic in the drinking water; however, these inclusions were mistakenly identified as micronuclei. To further examine the urothelial cell inclusions that occur in inorganic arsenic-exposed humans, we evaluated two patients with a history of acute promyelocytic leukemia treated for disease relapse with a combination of all-*trans* retinoic acid and arsenic trioxide. Posttreatment examination of the patients' urine cytology specimens by light and electron microscopy demonstrated cytoplasmic inclusions in exfoliated superficial urothelial cells similar to those seen in mice. The inclusions were present in decreasing quantities at 3 and 7 months after completion of treatment. No comparable inclusions were detected in exfoliated urothelial cells in urine from six individuals not treated with arsenic trioxide. Based on the results of the examination by light and electron microscopy, we have determined that urothelial cell inclusions in the urine of humans previously identified as micronuclei are instead intracytoplasmic inclusions similar to those found in arsenic-treated mice.

Key Words: **urothelial cell; arsenic trioxide, bladder.**

Inorganic arsenic (arsenite or arsenate) is a known human carcinogen for the urinary bladder, lung, and skin (NRC, 1999, 2001). In mouse models, intracytoplasmic inclusions have been observed within the urothelium of mice orally administered high doses of arsenite or arsenate. These inclusions were shown to be intramitochondrial by electron microscopy and contain arsenite bound to protein (Suzuki *et al.*, 2008). The inclusions do not appear to be necessary for the arsenic-induced cytotoxicity and regenerative hyperplasia that occur in the urothelium of treated mice because they do not occur in arsenic-treated rats that show similar cytotoxicity and regeneration. Instead, they may be a possible protective mechanism as has been seen with other metals such as lead (Brown *et al.*, 1985; Gonick, 2011; Masci *et al.*, 1995).

In humans, epidemiologic studies evaluating arsenic-related urothelial cytologic effects have reported the presence of cytoplasmic inclusions in exfoliated human urothelial cells in the urine of individuals exposed to high levels of arsenic in the drinking water (Ghosh *et al.*, 2008; Moore *et al.*, 1997). These inclusions have been classified as micronuclei. However, in some studies, micronuclei were identified based on positive staining with the nonspecific Giemsa stain, which has been shown to give false positive results in epithelial cells (Nersesyan *et al.*, 2006). In studies using centromere-specific stains, often the majority of inclusions identified as micronuclei were centromere negative (Ghosh *et al.*, 2008; Marchiset-Ferlay *et al.*, 2012; Moore *et al.*, 1997).

To evaluate the possibility that these inclusions occurring in arsenic-exposed humans are not micronuclei, but rather similar to the arsenic-containing granules observed in mice, we evaluated two patients with acute promyelocytic leukemia (APL) treated with arsenic-based chemotherapy. Both patients had intracytoplasmic inclusions in exfoliated urothelial cells present in their urine similar to those seen in the urothelium of mice. By light microscopy these inclusions were indistinguishable from what has been classified as micronuclei in epidemiologic studies of populations exposed to high concentrations of inorganic arsenic in the drinking water, but by electron microscopy we show that they are the same intracytoplasmic inclusions detected in mice and distinct from micronuclei. In addition, these same inclusions did not stain with the DNA-specific stain 4′,6-diamidino-2-phenylindole (DAPI).

MATERIALS AND METHODS

Chemical

Arsenic trioxide (ATO) (Cephalon Inc., Frazer, PA) was obtained from the Nebraska Medical Center inpatient pharmacy and administered iv at a dose of 0.15 mg/kg body weight per day to the APL patients. The study protocol was approved by the University of Nebraska Medical Center Institutional Review Board.

Control Subjects

Six healthy female volunteers, aged 25–69 years, were recruited from the student and employee population at the University of Nebraska Medical Center (UNMC) and the Nebraska Medical Center.

ATO-Treated Subjects

Case 1. The first patient is a 36-year-old female with a history of APL, which was first diagnosed at age 30. Initial cytogenetic analysis showed the classic t(15;17)(q22;q11.2) translocation. The patient entered complete remission after treatment with all-*trans* retinoic acid (ATRA) and daunorubicin, followed by consolidative therapy with cytarabine and ATO. ATO was administered iv at a dose of 0.15 mg/kg for 30 consecutive days. However, 6 years after entering remission, the patient was found to have leukopenia and thrombocytopenia, and a bone marrow biopsy showed disease relapse with hypercellular bone marrow (80%) composed of 53% blasts and promyelocytes. Concurrent flow cytometry completed on the bone marrow specimen was consistent with relapsed APL. The patient was subsequently treated with two courses of ATO (0.15 mg/kg, iv) for 30 consecutive days, each time with 3 weeks between the two courses. After treatment, the patient again reached complete remission and subsequently underwent autologous stem cell transplantation.

Case 2. The second patient is a 43-year-old male with a history of APL diagnosed at age 38. Initial cytogenetic studies revealed the presence of an abnormal diploid clone, which was characterized by a reciprocal translocation involving 15q and 17q, and two subclones; the first subclone was characterized by an interstitial deletion of 5q, and the second subclone was characterized by an interstitial deletion of 5q and additional unknown material on 7q. The patient received induction chemotherapy composed of anthracycline and ATRA, with consolidation therapy using idarubicin and cytarabine. Subsequently, the patient received maintenance therapy with ATRA, methotrexate, and 6-mercaptopurine for 2 years. On this regimen, the patient entered complete morphologic and molecular remission. However, 5 years after initial diagnosis, the patient was noted to have mild thrombocytopenia (platelet count: 140,000/μl [ref: 150,000–400,000/μl]) and underwent bone marrow biopsy, which showed disease relapse with normocellular bone marrow containing 8% blasts. Cytogenetic analysis showed identical derangements as those present in the original diagnostic sample. Flow cytometric studies were likewise consistent with relapsed APL. The patient was subsequently treated with ATO (0.15 mg/kg, iv) for 18 days, 5 days per week and again entered complete disease remission. He then underwent autologous stem cell transplantation.

Urine Samples

Control subjects. Random urines collected at various times during the day were collected from six control subjects. Samples were processed for examination by light and electron microscopy.

ATO-treated subjects. In the first case, random afternoon fresh void urine was collected after completion of 30 days of ATO treatment and again 3 and 7 months after termination of treatment. In the second case, a random afternoon fresh void urine specimen was collected after completion of 60 days of ATO treatment. Samples for both cases were processed for examination by light microscopy and transmission electron microscopy and, in the first case, by fluorescent microscopy.

Light Microscopy

One half of each urine sample was fixed in an equal volume of Shandon Cytospin Collection Fluid (Thermo Scientific, St Louis, MO). The preserved sample was stored at approximately 4°C until it was transported to the cytology laboratory for processing. Specimens were spun at 2000 rpm for 10 min, the supernatant was removed, and the cell button was resuspended in saline (two cycles). Then, three to four drops of the solution were placed into a plastic chamber holding a glass slide; the glass slide (in holder with solution) was spun at 2000 rpm for 5 min. The glass slide was then removed from the plastic chamber and stained with hematoxylin and eosin (H & E).

Fluorescent Microscopy

After examination by light microscopy, the H&E-stained slide from the urine sample collected in the first case after 30 days treatment with ATO was scanned and coordinates and images of cells containing inclusions were captured. The slide was destained to remove most of the H&E stain, restained with DAPI, and scanned using fluorescent microscopy. Based on the coordinates in the two scanned images, cells containing inclusions were identified.

Transmission Electron Microscopy

The remaining half of each urine sample was spun for 10 min at 1700 rpm, and the supernatant was removed. The remaining sediment was fixed in 5–10 ml EM fixative, (Millonig's Fixative (MPG), 2.0% paraformaldehyde/2.5% gluteraldehyde) and stored at approximately 4°C until it was transported to the EM laboratory for processing. Specimens were spun at 1700 rpm for 10 min in a plastic conical centrifuge tube to form a loose pellet of cells. Supernatant was removed and replaced with 300 μl of histogel (Thermo Scientific) at 65°C and allowed to cool to room temperature. The cell pellet was removed, dissected into 1-mm cubes, and postfixed in EM fixative at 4°C for 1 h. EM blocks were processed on an automated EM tissue processor (Leica EM TP, Buffalo Grove, IL). The blocks underwent secondary fixation with osmium tetroxide, after which they were washed in triple distilled deionized water for 10 min (x2). The tissue was then dehydrated with a series of ethyl alcohol dilutions, followed by treatment with acetonitrile (three changes for 5 min). The tissue was then infiltrated with fresh resin according to the following infiltration methods: 1:1 acetonitrile:resin for 1 h; fresh Poly/Bed 812 resin for 1 h. Tissue was removed from the processor and then placed in fresh Poly/Bed 812 resin to polymerize in an oven at 60°C for 48 h. Plastic survey thick sections were obtained at 1 μm, stained with 0.5% toluidine blue, and examined under the light microscope. Selected areas were ultrasectioned at 70–90 nm (silver sections), mounted on copper grids and stained with uranyl acetate and lead citrate, and examined in the electron microscope (JEOL 1230, Peabody, MA).

RESULTS

Light Microscopic Examination

Control subjects. No inclusions were observed in the urothelial cells present in the urine of control subjects when examined by light microscopy.

ATO-treated subjects. By light microscopy, in both cases, round, eosinophilic-to-deep blue intracytoplasmic inclusions were detected in most exfoliated superficial urothelial cells in the specimens collected at the end of treatment, often with many inclusions of various sizes present in individual cells. There was no evidence of cellular necrosis or apoptosis (Fig. 1A). In case 1, in urine samples collected approximately 3 and 7 months after ATO treatment was stopped, the intracytoplasmic inclusions were still present in the exfoliated superficial urothelial cells but were fewer in numbers in individual cells,

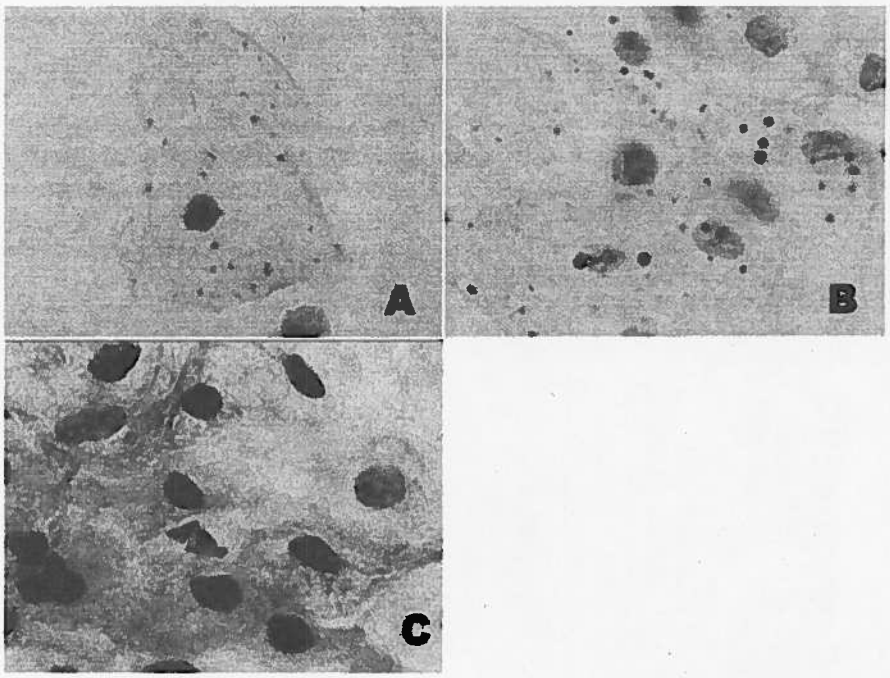

FIG. 1. Light microscopy (H & E). Urothelial cells showing scattered, eosinophilic-to-deep blue intracytoplasmic inclusions after 30 days of therapy (A) and again 3 months after termination of treatment (B). Urothelial cells from control case (C) (×1000).

and inclusions were not present in all of the cells (Fig. 1B). In the 7-month posttreatment urine specimen, only 10–20% of the exfoliated urothelial cells contained inclusions.

Fluorescent Microscopy

Intracytoplasmic inclusions present in the exfoliated urothelial cells in urine collected after 30 days treatment with ATO in case one did not stain with the DNA-specific stain DAPI (Fig. 2).

Transmission Electron Microscopy Examination

Control subjects. No abnormalities were observed by TEM in the urines from control subjects.

ATO-treated subjects. Investigation by TEM of cell pellets from all time points in case 1 and from case 2 showed that the intracytoplasmic inclusions were round, present in membrane-bound round organelles, and varied in size. Although we were not able to definitively ascertain in which organelle the granules were present, they appeared to be present in lysosomes (Fig. 3).

DISCUSSION

Pharmacologic use of arsenic-based chemotherapy was common in the mid-1800s to the early 1900s but fell out of favor with the advent of less-toxic therapies (Kwong and Todd, 1997). More recent studies have shown the efficacy of treatment using ATO treatment in patients with APL, reviving its medical use (Chen *et al.*, 1997; Shen *et al.*, 1997). However, exposure to high levels of environmental inorganic arsenic is a known cause of multiple cancers in humans, including urothelial carcinoma (Smith *et al.*, 1992; Wu *et al.*, 1989).

Exposure to high concentrations of other heavy metals, such as lead, results in their concentration within mitochondria (Brown *et al.*, 1985; Gonick, 2011). Our prior studies investigating the mechanism of arsenic-induced urothelial cytotoxicity using rat and mouse models demonstrated intramitochondrial inclusions containing inorganic arsenic within the urothelium of mice treated with high levels of inorganic or organic arsenic; the same inclusions were not seen in the urothelium of similarly treated rats (Suzuki *et al.*, 2008). The major form of arsenic found in the mouse inclusions was inorganic arsenite similar to the results Hernández-Zavala *et al.* (2008) found when they analyzed the arsenic content of exfoliated urothelial cells in the urine of humans exposed to arsenic in the drinking water (< 1 to 190 µg arsenic/l).

We observed inclusions similar to those in mice by light and transmission microscopy in exfoliated human urothelial cells in the urine of two patients treated with ATO. The inclusions were not associated with signs of necrosis or apoptosis, suggesting that they represent a cellular depot collection of bound arsenite not associated with toxicity. This conclusion is further supported by their continued presence 3 and 7 months after cessation of treatment. The urothelium is a slowly proliferating tissue in most species with turnover times estimated at 6 months to 2 years (Rebel *et al.*, 1994; Tiltman and Friedell, 1972). It appears that the inclusions remain until the superficial cells are exfoliated as part of the normal turnover of the urothelium. Therefore, the presence of these inclusions in exfoliated

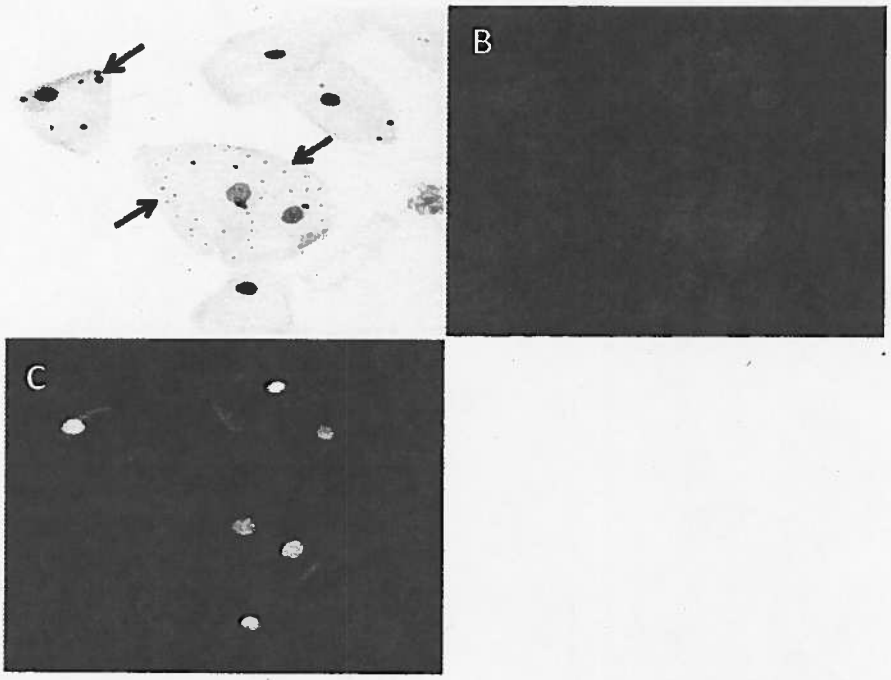

FIG. 2. Fluorescent microscopy. (A) A black and white micrograph showing intracytoplasmic inclusions (arrows) present in the exfoliated urothelial cells present in the urine after 30 days of treatment with ATO. (B) DAPI staining (blue) of the same area of the slide showing positive staining only of the cell nuclei but no staining of the intracytoplasmic granules. (C) A composite photograph of the H & E stain and the DAPI stain.

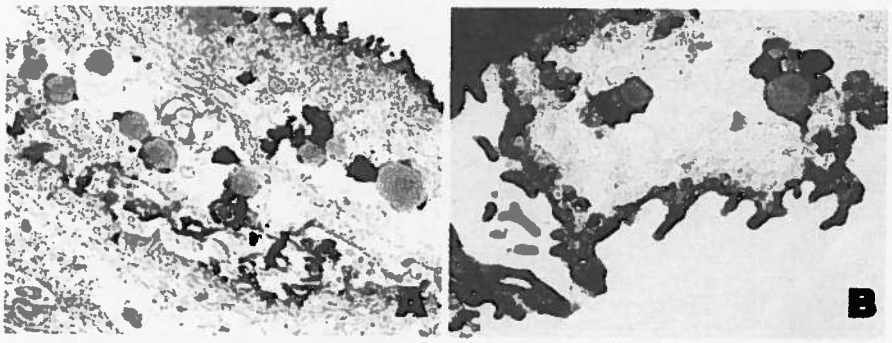

FIG. 3. Electron microscopy. Exfoliated urothelial cells present in the urine 3 months after termination of therapy showing membrane-bound spherical inclusions apparently in the lysosomes. ×3000 (A). The inclusions at higher magnification. ×5000 (B).

urothelial cells in the urine provides a marker of high exposure to inorganic arsenic, whether from environmental exposure or from arsenic-containing pharmaceuticals in a specimen which can be obtained by noninvasive techniques.

In mice, the urothelial inclusions occur in the mitochondria (Suzuki *et al.*, 2008). In mouse studies, the urothelium is well preserved as the bladder is inflated with fixative while the animal is under deep anesthesia, preventing autolysis (Suzuki *et al.*, 2008). Under such circumstances, the organelle structures are well preserved, making it possible to identify the presence of the inclusions in mitochondria. In the present studies in humans, the cells are exfoliated superficial cells present in urine and have degenerative changes associated with autolysis. Nevertheless, the overall impression by electron microscopy is that the inclusions are present in lysosomes in these human specimens, in contrast to their location in mitochondria in mice. However, the precise location of these inclusions in the human cells requires further investigation.

Previous studies have shown an increased incidence of cytoplasmic inclusions classified as micronuclei in exfoliated bladder cells from individuals exposed to high levels of inorganic arsenic in the drinking water, suggesting possible genotoxicity (Ghosh *et al.*, 2008). In some studies, the apparent micronuclei were detected using the Giemsa stain, a nonspecific stain which has been shown to increase false positive identification of micronuclei (Nersesyan *et al.*, 2006). In this study, we

showed that these cytoplasmic inclusions do not stain when the DNA-specific stain DAPI is used. The intracytoplasmic inclusions observed in the urothelial cells of arsenic-treated mice also do not stain with DAPI (unpublished observations). In other studies, centromere markers were used, but most of the increase in apparent micronuclei were in centromere-negative micronuclei (Ghosh *et al.*, 2008; Marchiset-Ferlay *et al.*, 2012; Moore *et al.*, 1997). Some micronuclei are present in urothelial cells normally as in most cells (Nersesyan *et al.*, 2006). Because the micronuclei and cytoplasmic arsenic inclusions have an identical appearance by light microscopy, it is likely that most if not all of these centromere-negative inclusions are actually inclusions composed of arsenic. The appearance by electron microscopy of the inclusions in the cases we examined show that they are contained in membrane-bound organelles and do not have the appearance of micronuclei.

In conclusion, based on the results of the examination by light, fluorescent, and electron microscopy, we have determined that urothelial cell inclusions in the urine of humans exposed to high levels of arsenic previously identified as micronuclei are instead intracytoplasmic inclusions similar to those found in arsenic-treated mice.

FUNDING

Arsenic Science Task Force.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Sharon Auw and Cheryl Putnam with the preparation of this manuscript.

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Arch Toxicol
DOI 10.1007/s00204-014-1305-8

GENOTOXICITY AND CARCINOGENICITY

Lung tumors in mice induced by "whole-life" inorganic arsenic exposure at human-relevant doses

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Received: 24 January 2014 / Accepted: 23 June 2014

Abstract In mice, inorganic arsenic in the drinking water in the parts per million range via the dam during in utero life or with whole-life exposure is a multi-site carcinogen in the offspring. However, human arsenic exposure is typically in the parts per billion (ppb) range. Thus, we studied "whole-life" inorganic arsenic carcinogenesis in mice at levels more relevant to humans. Breeder male and female CD1 mice were exposed to 0, 50, 500 or 5,000 ppb arsenic (as sodium arsenite) in the drinking water for 3 weeks prior to breeding, during pregnancy and lactation, and after weaning (at week 3) groups of male and female offspring (initial $n = 40$) were exposed for up to 2 years. Tumors were assessed in these offspring. Arsenic exposure had no effect on pregnant dam weights or water consumption, litter size, offspring birthweight or weight at weaning compared to control. In male offspring mice, arsenic exposure increased ($p < 0.05$) bronchiolo-alveolar tumor (adenoma or carcinoma) incidence at 50-ppb group (51 %) and 500-ppb group (54 %), but not at 5,000-ppb group (28 %) compared to control (22 %). These arsenic-induced bronchiolo-alveolar tumors included increased ($p < 0.05$) carcinoma at 50-ppb group (27 %) compared to controls (8 %). An increase ($p < 0.05$) in lung adenoma (25 %) in the 50-ppb group compared to control (11 %) occurred in female offspring. Thus, in CD1 mice whole-life arsenic exposure induced lung tumors at human-relevant doses (i.e., 50 and 500 ppb).

Keywords Arsenic · Carcinogenesis · Mice · Whole-life exposure · Lung cancer

Introduction

Inorganic arsenic is a multi-site human carcinogen with targets including the lung, skin and urinary bladder, and drinking water is a major route of exposure (IARC 2004, 2012). Estimates place tens of millions of people worldwide at risk for chronic exposure to potentially harmful levels of inorganic arsenic primarily through drinking water (Naujokas et al. 2013). In humans, in utero or early life inorganic arsenic exposure from contaminated drinking water has been associated with subsequent lung cancer in adulthood (Smith et al. 2006; Marshall et al. 2007) or other cancers (Smith et al. 2012; Liaw et al. 2008; Yuan et al. 2010) recognized as targets of inorganic arsenic in humans (IARC 2004, 2012). Similarly, in mice, the lung is a common target site after in utero exposure via inorganic arsenic in the maternal drinking water (Waalkes et al. 2003, 2004, 2006a, b; Tokar et al. 2012). Whole-life inorganic arsenic exposure

Electronic supplementary material The online version of this article (doi:10.1007/s00204-014-1305-8) contains supplementary material, which is available to authorized users.

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Published online: 09 July 2014

in mice, which includes gestational exposure, produces a generally similar spectrum of tumors as in utero exposure, only at lower doses, and includes dose-related increases in lung cancers (Tokar et al. 2011). Thus, it appears inorganic arsenic exposure during development stimulates lung carcinogenesis that manifests in adulthood in both humans and rodents (Smith et al. 2006; Marshall et al. 2007; Waalkes et al. 2003, 2004, 2006a, b; Tokar et al. 2012), although most human populations are generally exposed during their whole life (IARC 2004, 2012).

Thus far, for in utero exposures to inorganic arsenic to result in adulthood cancer in mice, the exposure levels used in the maternal drinking water have been in the moderate parts per million (ppm) level (42.5 or 85 ppm; Waalkes et al. 2003, 2004, 2006a, b; Tokar et al. 2012), while human arsenic-associated cancers generally occur with exposures in the parts per billion (ppb) range (for examples see: IARC 2004, 2012). We developed a "whole-life" exposure model in mice which involves inorganic arsenic exposure via the drinking water to breeders 3 weeks prior to breeding, to the dams during pregnancy and lactation, and then to the offspring after weaning through adulthood to about 2 years of age (Tokar et al. 2011). With this whole-life exposure, lower inorganic arsenic doses result in tumor formation as in, for instance, lung cancers that occur to excess at 24 ppm but match the control rates at 6 ppm (Tokar et al. 2011). Rodent tumor endpoint assays can be insensitive, and their sensitivity can vary with a variety of parameters such as group size. Moreover, some have proposed that arsenic has complex dose–response characteristics with biological activity, like steroid receptor gene activation, that occurs at low doses but is lost at higher doses (Bodwell et al. 2004, 2006). This dose–response dichotomy seems to exist within oncogenesis as inorganic arsenic is a very effective cancer chemotherapeutic at pharmacologic doses, but can also enhance xenograft tumor growth and angiogenesis at lower doses (Soucy et al. 2003; Kamat et al. 2005; Liu et al. 2006). In addition, functional or genetic changes relevant to chronic lung disease and cancer have been reported after exposure in early life (including in utero exposure) to inorganic arsenic at doses as low as 100–500 ppb in rodents (Petrick et al. 2009; Lantz et al. 2009; Kozul et al. 2009; Ramsey et al. 2013a, b; Farzan et al. 2013).

Therefore, we designed the present study to expose mice to inorganic arsenic throughout gestation and then for the bulk of their adult life to a range of arsenic levels encompassing much lower doses such as those encountered commonly in the human environment. The original intent of this work was to provide rodent data potentially useful in more clearly defining levels of concern for inorganic arsenic. We used our "whole-life" protocol that exposes mice to inorganic arsenic via the drinking water through their entire in utero life stage and subsequently through adulthood until 2 years of age (Tokar et al. 2011) as this would duplicate how humans most likely would be exposed via the environment. The lower two dosage levels of drinking water inorganic arsenic used in the present study (50 and 500 ppb arsenic) are much more commonly seen in human drinking water (IARC 2004, 2012), and the lowest level (50 ppb) approaches the USEPA maximum contaminant level in drinking water for arsenic in the United States (10 ppb).

Materials and methods

Animals and treatment

This study started in June of 2009. The animal portion of the present study was designed and performed entirely within the National Cancer Institute facility at Fort Detrick in Frederick, MD. Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996). The NCI-Frederick animal facility and its animal program are accredited by the American Association for Accreditation of Laboratory Animal Care, and the animals were treated humanely and with regard for alleviation of suffering. The CD1 mice used for breeders and for initial dosing were obtained from Charles River South (Raleigh, NC). Mice were housed under conditions of controlled temperature, humidity and light cycle.

A basal diet (5L79; Ralston Purina, St. Louis, MO) and acidified water were provided ad libitum. The chow used was not archived but is made of the same components as Purina's certified rodent diet which in its most recent testing was listed as below detection limits in total arsenic (<20 ppb; 2007; http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents). In June 2013, we tested 5L79 diet sent to NIEHS from the Fort Detrick animal facility building where this bioassay was performed. We found arsenic to be below the level of detection by graphite furnace-atomic absorption spectrometry (GF-AAS; model AAnalyst 600 PerkinElmer with AS 800 auto sampler and End-capped THGA graphite tubes) after digestion of the feed in 50 % of a 2:1 ultrapure nitric acid (70 %; Aldrich, St. Louis, MO): perchloric acid (70 %; Aldrich) mixture and 50 % water. By a Method Detection Limit Study for our GF-AAS, we could consistently detect 5 ppb of inorganic arsenic standard. A mixture of palladium and magnesium was used as a matrix modifier to prevent premature volatilization before vaporization and therefore loss of signal (IARC 2004). Recovery was 99–101 % for spiked arsenic in digested diet samples ($n = 8$) indicating minimal matrix effects. Although the bioavailability of arsenicals from the diet can be highly variable depending on the arsenical form, our data suggest that any contributions from

the feed were negligible compared to administered doses in the drinking water. Thus, the precise amount and form of arsenic that the diet added to the exposure in the present study is not known but is likely only a small fraction of the intentional exposure of inorganic arsenic from the drinking water. In addition, the dietary exposure would be equal between control and treated animals.

The water for this study was from the Fort Detrich water system. It is tested yearly for arsenic and is consistently reported to have non-detectable arsenic levels in compliance with USEPA and Maryland Department of the Environment testing regulations and limits and was so during the period of this study (<0.5 ppb by ICP-MS; personal communication, M. Schrader, NCI-Frederick). For arsenic treatment, sodium arsenite ($NaAsO_2$; purity 99 %; Sigma Chemical Co., St. Louis, MO) was added to the drinking water. The levels added were 0 (control), 50, 500 and 5,000 ppb arsenic.

The CD1 mouse (Charles River) strain was used. Breeder males (10 per dose) and females (20 per dose) received arsenic in the drinking water for at least 3 weeks before successful breeding. Once pregnant, dams received arsenic during the entire course of pregnancy, and after birth, the mothers received arsenic in the drinking water through the period of lactation. At weaning (3 weeks of age), offspring in initial groups of 40 per dose continued to receive arsenic in the drinking water through adulthood to planned killing at 104 weeks (birth was considered time = 0). This treatment is termed "whole life" although it did not go to the natural end of life for the individual animal. Tumors were assessed only in the offspring. At birth, litters were culled to no more than eight pups, consisting of four males and four females if possible. At weaning, offspring were randomly selected to continue in the offspring treatment groups, but it was made sure that they represented animals from atleast 10 litters treated with the same dosage. Within each treatment group, mice were allocated for further treatment into adulthood by random selection of 3–4 offspring mice/sex/litter from 10 to 12 litters of the 20 possible litters in each group.

Clinical data and pathological assessment

Individual offspring body weights were recorded once per week for the first 10 weeks after weaning and then once every month. Clinical signs were checked daily. Mice were killed with CO_2 when moribund or at the end of the study. A complete necropsy was performed on all moribund animals, animals found dead or at the end of the study. Tissues taken and processed for histological analysis included liver, kidneys, lungs, adrenals, spleen, thymus, thyroid, pituitary, urinary bladder, gonads (testes, ovary), uterus, vagina, oviduct, mammary gland, skin, brain and all grossly abnormal tissues. The skin and paws were carefully inspected for any lesions. Tissues were fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological analysis. The urinary bladder was inflated with fixative prior to being embedded. The pathologists were unaware of the treatment group during pathological assessments. Pathological peer review was conducted and provided consensus diagnoses. Significant increases were seen in lung tumors in several arsenic-treated groups. In order to expedite the reporting of these data, only primary lung lesions are reported in this work. There were tumors and non-neoplastic lesions in other tissues, but in no other site were tumors found to be related to arsenic exposure. Non-pulmonary lesions will be reported in a future publication.

Pathological evaluations and diagnoses of lung tumors and associated lung lesions were evaluated and diagnosed using standard diagnostic criteria as outlined in the International Harmonization of Nomenclature and Diagnostic Criteria in rats and mice (INHAND) document for respiratory tract lesions (Renne et al. 2009) in addition to criteria outlined in a descriptive document on the morphology of spontaneous and chemically induced pulmonary lesions in rats and mice (Dixon et al. 2008). Diagnostic criteria for lung bronchiolo-alveolar adenoma and bronchiolo-alveolar carcinoma included typical morphologic characteristics used for tumor diagnosis such as cell size and shape, degree of cellular atypia, cellular pleomorphism, invasive properties of tumor cells, tumor growth pattern and mitotic activity.

Data analysis

Data are given as lesion incidence (number of affected mice/total mice examined) or survival rate or as mean ± standard deviation or standard error of the mean (SEM), as appropriate. Data on males and females were analyzed separately. A probability level of $p < 0.05$ was considered to indicate a significant difference in all cases. The survival analyses excluded those animals that were found dead and too autolyzed to assess for lung tumors. In the analyses of tumor incidence, litter effects and survival were taken into account by use of a mixed effects logistic regression model that included survival time and a normally distributed random litter effect. Tumor incidence analyses p values were one-sided. Tumor incidence analyses is based on numbers of animals available for pathological examination, and loss of animals to observation was typically due to post-death autolysis that was considered too advanced for appropriate diagnosis. Lung tumors were considered part of a pathological continuum (adenoma and carcinoma) and were combined for analysis as "adenoma or carcinoma." When a separate lung adenoma and carcinoma occurred in a single mouse, it is counted as a single case in this either/

Table 1 Impact of arsenic exposure on CD1 mice until weaning

Measurements	Group (ppb)			
	Control	50	500	5,000
Dams used to make groups on day 21[a], *n*	12	11	11	11
Maternal body weights (g), *n*	12	11	11	11
Gestation day 10	35.0 ± 1.2	35.3 ± 1.1	35.4 ± 0.8	34.7 ± 0.9
Day 14	45.1 ± 1.5	44.9 ± 1.5	44.7 ± 1.2	44.9 ± 0.7
Day 18	59.8 ± 2.0	57.4 ± 3.8	60.4 ± 1.8	61.6 ± 1.1
Dam water consumption[b], *n*	9	11	10	8
Gestation day 16–18 (ml/dam/day)	12.6 ± 0.8	13.8 ± 1.0	13.8 ± 0.8	12.2 ± 0.7
Newborns/litter at birth[c], *n*	12	11	11	11
	12.3 ± 2.5	12.7 ± 0.8	12.2 ± 0.7	13.2 ± 0.5
Newborn body weight (g)[c], *n*	12	11	11	11
	1.73 ± 0.03	1.78 ± 0.05	1.81 ± 0.04	1.74 ± 0.05
Offspring body weights (g)				
Males day 21, *n*	10	10	11	11
	15.3 ± 0.5	16.3 ± 0.5	14.9 ± 0.5	15.4 ± 0.5
Females day 21, *n*	12	11	10	10
	14.3 ± 0.4	15.4 ± 0.3	14.6 ± 0.5	14.6 ± 0.4

Arsenic was given continuously as sodium arsenite in the drinking water to breeding pairs (10 males, 20 females) prior to breeding for 3 weeks, to pregnant dams, to lactating dams after birth (day 0), and then to the offspring from weaning up to 2 years of age when the experiment was terminated (see "Materials and methods"). Data are expressed as the mean ± SEM. No significant differences from control occurred ($p > 0.05$) in any of these metrics

[a] Litters used to constitute groups of original $n = 40$ groups of male or female offspring

[b] Water consumption data were based on slightly fewer dams than other metrics because water was released from the bottles by animals activity (stuffing bedding into water tube, etc.)

[c] Day 0. All newborns were born alive

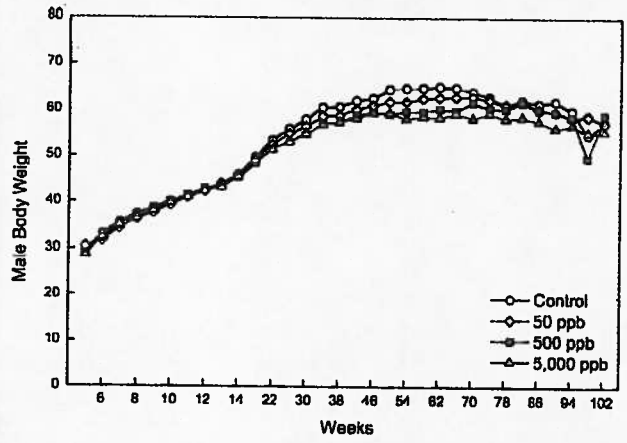
or category and noted as co-occurring in the "multiple tumors" category. There were also several instances of multiple primary lung adenoma in individual mice, and these are also discussed as cases of multiple tumors. For multiple comparisons of maternal body weight during pregnancy, maternal water consumption during pregnancy, average litter size at birth, newborn pup weight at birth and male or female weanling body weight (day 21), an ANOVA followed by a two-sided Dunnett's test was used. These analyses of measurements prior to and at weaning used the litters as the basis of comparison and first averaged within litters then averaged these litter means for summary statistics. Survival analyses were performed by Cox regression and Tarone's test according to sex and arsenic dose, and all p values for these tests are two-sided. Individual adult body weights were analyzed by repeated-measures ANOVA followed by a two-sided Dunnett's test.

Results

Male and female breeder CD1 mice were exposed to 0, 50, 500 or 5,000 ppb arsenic in the drinking water at least 3 weeks prior to successful breeding. Arsenic exposure continued for the mothers during pregnancy and lactation and for the individual offspring in gender-based groups after weaning (at 3 weeks of age) until 2 years of age (see "Materials and methods"). Tumors were assessed in the offspring. This protocol was termed "whole-life" arsenic exposure although it did not go to the natural end of life of the individual mouse in all cases. All tumor incidence data comparisons are adjusted for survival and litter effects (see "Materials and methods").

Survival and body weights

The arsenic exposure during early life did not impact maternal water intake or maternal body weight growth during pregnancy (Table 1). In utero fetal growth was unaffected by these levels of arsenic exposure as the number of newborns/litter and newborn body weight did not differ based on arsenic treatment. Trans-lactational arsenic exposure after gestational exposure via the dam to these levels of arsenic had no effect on neonatal growth as assessed by offspring body weight at weaning (Table 1).



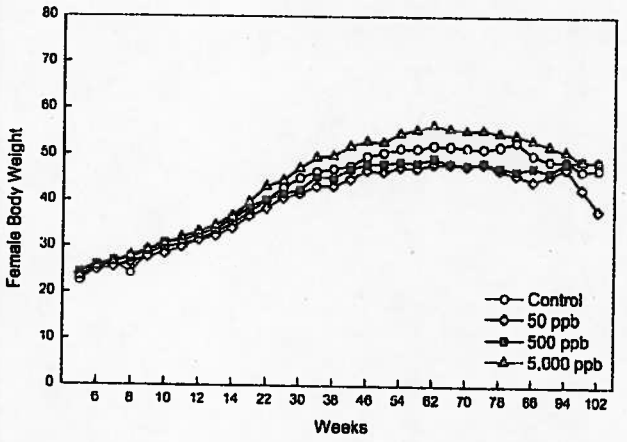
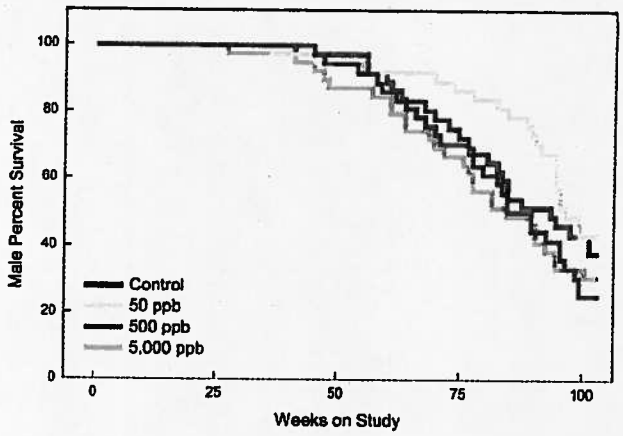


Fig. 1 Body weights during adulthood in male (*top*) and female (*bottom*) CD1 mice exposed to whole-life inorganic arsenic. See "Materials and methods" for details of exposure. Standard deviations are omitted for clarity. Because of concurrent unrelated demands on trained necropsy personnel, terminal killing was staggered over week 104 and 105, and the week 104 body weights would not be a true time-based mean and are not included. The occasional differences that occurred in treated animals compared to control rarely exceed 10 % and are detailed in the "Results" text. For precise numerical values, see Supplemental Tables 1 and 2



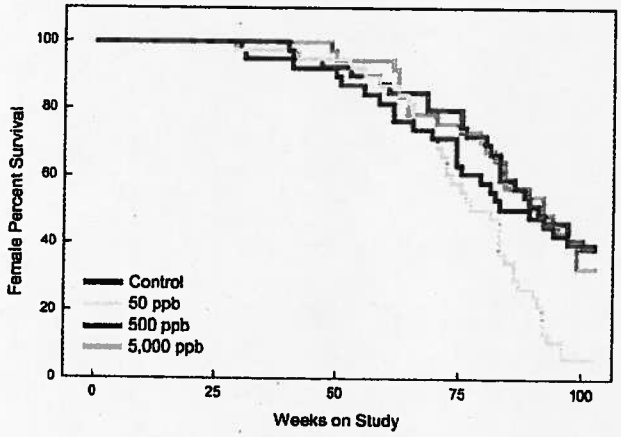


Fig. 2 Kaplan–Meier survival curves for male (*top*) and female (*bottom*) CD1 mice exposed to whole-life inorganic arsenic. Survival was reduced in females exposed to 50 ppb. See Table 2 for details and statistical analyses

Offspring body weights during adulthood are shown in Fig. 1 (numerical data are available in Supplemental Table 1 and 2). In the adult male offspring, exposure to arsenic had some transient effects (weeks 50–74) on adult offspring body weights but only in the groups exposed to the highest two doses (500 and 5,000 ppb). Body weights were significantly ($p < 0.05$) reduced in male offspring exposed to 500 or 5,000 ppb as assessed on weeks 50, 54, 58, 62, 66 and in the 5,000-ppb group additionally on weeks 70 and 74 but returned to control levels for the remainder of the study (Supplemental Table 1). Only in one case did these differences exceed 10 % of the control mean, specifically on week 62 when the difference between control (average 65.2 g) and 5,000-ppb-treated male mice (average 58.4 g) was 10.4 %. In male offspring treated with the lowest arsenic dose (50 ppb), there were no significant differences in body weight compared to control at any time in the study. In female offspring, reduced post-weaning body weights occurred only at 78 and 82 weeks for the group receiving 50 ppb compared to control (Supplemental Table 2). Increased body weight compared to control occurred at 62 weeks of treatment for the group receiving 5,000 ppb. The lack of consistent body weight differences in arsenic-treated females makes these changes appear unrelated to treatment.

Survival of progeny mice is shown in Fig. 2 and Table 2. There were no significant differences in survival from control in the males treated with any of the levels of arsenic. Control male average survival in the present study (595 days or 85 weeks) is essentially the same as the survival seen in control males in our prior whole-life study (85 weeks; Tokar et al. 2011). In females, the 5,000 and 500 ppb doses had no impact on survival, but there was a significant reduction in survival for the mice treated with the lowest dose of arsenic (50 ppb) compared to females

Table 2 Survival after whole-life inorganic arsenic exposure in mice evaluated for lung cancer

Arsenic concentration	Control	50 ppb	500 ppb	5,000 ppb
Males				
Group size examined[a]	36	37	37	39
Moribund/early deaths	27	23	23	27
Mice surviving to termination	9	14	14	12
Percent survival at termination (%)	25	38	38	31
Mean survival (days)	595	638	608	573
Survival analysis p values[b]	0.375 (trend)	0.126	0.352	0.960
Females				
Group size examined[a]	38	38	39	37
Moribund/early deaths	23	36	24	25
Mice surviving to termination	15	2	15	12
Percent survival at termination (%)	39	5	38	32
Mean survival (days)	573	548	604	611
Survival analysis p values[b]	0.260 (trend)	0.008**	0.771	0.983

Arsenic was given continuously as sodium arsenite in the drinking water to breeding pairs prior to breeding for 3 weeks, to pregnant dams, to lactating dams after birth (day 0), and then to the offspring from weaning up to 2 years of age when the experiment was terminated (see "Materials and methods"). Tumors were only assessed in the offspring. Data are expressed as the mean survival days (% evaluated). Initial group size was 40. Groups were derived from 10 to 12 litters (see Table 1 for details)

** Significant difference from control at $p < 0.01$

[a] Survival is based on mice examined for lung tumors. The difference between the original group size ($n = 40$) and the group size examined is due to animals lost to observation (found dead and too autolytic for evaluation). No significant differences from control occurred in survival in males ($p > 0.05$). Females exposed to the 50-ppb level showed a significant suppression of survival that did not occur at higher levels of exposure

[b] The p values under control are for dose-related trend in survival, which were not significant for either sex

Table 3 Bronchiolo-alveolar tumors and proliferative lesions induced by whole-life arsenic exposure in males

Lesion	Lesion-bearing mice/total examined	Percent (%)	p value
Adenoma			
Control	5/36	14	–
50 ppb	10/37	27	0.184
500 ppb	14/37	38	0.024*
5,000 ppb	6/39	15	0.415
Trend p			0.233
Carcinoma			
Control	3/36	8	–
50 ppb	10/37	27	0.037*
500 ppb	7/37	19	0.112
5,000 ppb	6/39	15	0.166
Trend p			0.487
Adenoma or carcinoma			
Control	8/36	22	–
50 ppb	19/37	51	0.023*
500 ppb	20/37	54	0.005**
5,000 ppb	11/39	28	0.230
Trend p			0.196
Multiple lung tumors[a]			
Control	1/36	3	–
50 ppb	5/37	14	–
500 ppb	2/37	5	–
5,000 ppb	1/39	3	–
Alveolar hyperplasia			
Control	1/36	3	–
50 ppb	4/37	11	–
500 ppb	2/37	5	–
5,000 ppb	1/39	3	–

Arsenic was given continuously in the drinking water throughout life. Sample size is the number of mice available for pathological analysis. Data are expressed as mice with a given lesion (% pathologically assessed). Groups were derived from 10 to 11 litters with original $n = 40$ individuals (see Table 1 for details)

* Significant difference from control at $p < 0.05$

** Significant difference from control at $p < 0.01$. Data analyses are adjusted for survival and litter effects

[a] One mouse in each treatment group had both a lung carcinoma and an adenoma. This is counted as a single event in "carcinoma or adenoma" and an event in "multiple tumors"

controls. Most of these deaths in the female 50-ppb group occurred relatively late in the study (>70 weeks). The reasons for the loss of females at the lowest dose are unclear.

Lung tumors and other lung lesions

Table 3 shows the effect of whole-life arsenic exposure on incidence of lung tumors in male mice. In males, whole-life arsenic exposure induced significant increases ($p < 0.05$) in bronchiolo-alveolar tumor (adenoma or carcinoma) incidence at 500 ppb (54 %) and 50 ppb (51 %) but not at 5,000 ppb (28 %), compared to control incidence (22 %). Arsenic-induced bronchiolo-alveolar tumors included significantly increased ($p < 0.05$) carcinoma at 50 ppb (27 %) compared to controls (8 %). At the 500-ppb level, adenoma incidence (38 %) was significantly increased ($p < 0.05$) over the control rate (14 %).

In male mice, the first lung tumor, a bronchiolo-alveolar carcinoma, was observed at 46 weeks in a mouse treated with 5,000 ppb arsenic and was the cause of early termination. All male control mice survived to this point in the study.

The control rates in male CD1 mice of lung adenoma (14 %), carcinoma (8 %) and adenoma or carcinoma (22 %) in the present study were comparable with control lung tumor rates from our most recent tumor endpoint study concerning inorganic arsenic using CD1 male mice from the same source and conducted at the same facility (adenoma, 14 %; carcinoma, 6 %; adenoma or carcinoma, 20 %; Tokar et al. 2012).

In arsenic-treated males, multiple lung tumors (multiple primaries in a single mouse) did not show significant differences but occurred to a numerically greater extent in the 50 ppb (14 %) compared to control (3 %) or the other arsenic treatment groups (5,000 ppb, 3 %; 500 ppb, 5 %; Table 3). Also in the 50-ppb group, one case of multiple lung tumors was a case of multiple adenomas along with a carcinoma. The 50-ppb group also showed numerically more cases of alveolar hyperplasia (11 %) than control (3 %) or the other arsenic treatment groups (5,000 ppb 3 %; 500 ppb, 5 %).

Bronchiolo-alveolar adenomas when compared to normal lung alveolar regions consisted of well-circumscribed areas of cuboidal tumor cells that tended to fill adjacent alveolar spaces. The benign tumor cells were relatively uniform, had little to no pleomorphism or atypia and had very low mitotic activity. The lung adenomas often compressed the surrounding tissue; however, there was no invasion, with defined borders that demarcated the tumor cells from the surrounding tissue. In contrast, the lung bronchiolo-alveolar carcinomas consisted of irregular to poorly circumscribed areas of tumor cells that occupied large regions of lung lobes and caused architectural distortion of the surrounding tissue. The malignant tumor cells were pleomorphic, ranging from low cuboidal to columnar with increased mitotic activity, and often formed papillary structures. The lung carcinoma cells were infiltrative and had ill-defined borders. Histopathological images of the various arsenic-induced bronchiolo-alveolar tumors compared to a control lung in males are shown in Fig. 3a–e. The representative images contain both low and high magnifications of a control lung (Fig. 3a, b), and an adenoma. (Fig. 3c, d) and a carcinoma (Fig. 3e, f) from male mice exposed to 50 ppb arsenic. Criteria used for tumor diagnosis are provided in the Methods. The large lung carcinomas often nearly filled an entire lung lobe (Fig. 3e).

Pulmonary non-neoplastic lesions occurred in male mice but with no association with arsenic treatment. This included one case of bronchus epithelial hyperplasia each in the 5,000 and 50-ppb groups, one case of bronchiole epithelial hyperplasia in the 500-ppb group, one case of bronchus epithelial hyaline accumulation in the 50-ppb group, one case of lymphoid hyperplasia in controls, one in the 500-ppb group and three in the 5,000-ppb groups, and one case of osseous metaplasia in the 50-ppb group. Lung fibrosis (2 cases in control, 5 in the 50-ppb group, 3 in the 500-ppb group and 5 in the 5,000-ppb group), alveolar histiocytic infiltration (10 cases in control, 13 in the 50-ppb group, 14 in the 500-ppb group and 13 in the 5,000-ppb group) and hemorrhage (one case in control, 2 cases in the 50-ppb group, 2 in the 500-ppb group and one in the 5,000-ppb group) occurred as well but were not related to treatment.

In females, a significant ($p < 0.05$) increase (Table 4) in lung adenoma occurred at 50-ppb group (25 %) compared to control (11 %). Carcinoma and adenoma or carcinoma showed no treatment-related differences from control with arsenic exposure in female offspring. One female mouse in the 5,000-ppb treatment group had a case of multiple adenoma. Alveolar hyperplasia was unremarkable in females (Table 4).

The control rates of lung adenoma (11 %), carcinoma (5 %) and adenoma or carcinoma (16 %) in female CD1 mice in the present study were comparable with control lung tumor rates from our most recent tumor endpoint study concerning inorganic arsenic using CD1 female mice from the same source and conducted at the same facility (adenoma, 14 %; carcinoma, 6 %; adenoma or carcinoma, 20 %; Tokar et al. 2011).

Pulmonary non-neoplastic lesions occurred in female mice with no association with arsenic treatment. This included one case of chronic pleura inflammation in the 500-ppb group; one case of intima vascular proliferation in the 5,000-ppb group; one case of hemorrhage each in the 50-ppb, 500-ppb and 5,000-ppb groups; one case of vascular inflammation in control group; and one case of mesothelial hyperplasia in the 500-ppb group. Lung fibrosis (3 cases in control, 2 in the 50-ppb group, 3 in the 500-ppb group and 5 in the 5,000-ppb group), alveolar histiocytic infiltration (7 cases in control, 7 in the 50-ppb group, 8 in the 500-ppb group and 13 in the 5,000-ppb group), and lymphoid hyperplasia (7 cases in control, 6 in the 50-ppb group, 7 in the 500-ppb group and 6 in the 5,000-ppb group) occurred but were unrelated to treatment.

Discussion

The whole-life exposure mouse model for inorganic arsenic in the drinking water generally duplicates more typical human environmental arsenic exposure (IARC 2004, 2012). In prior work, use of this whole-life drinking water inorganic arsenic exposure model in CD1 mice used drinking water levels ranging from 6 to 24 ppm (Tokar et al. 2011),

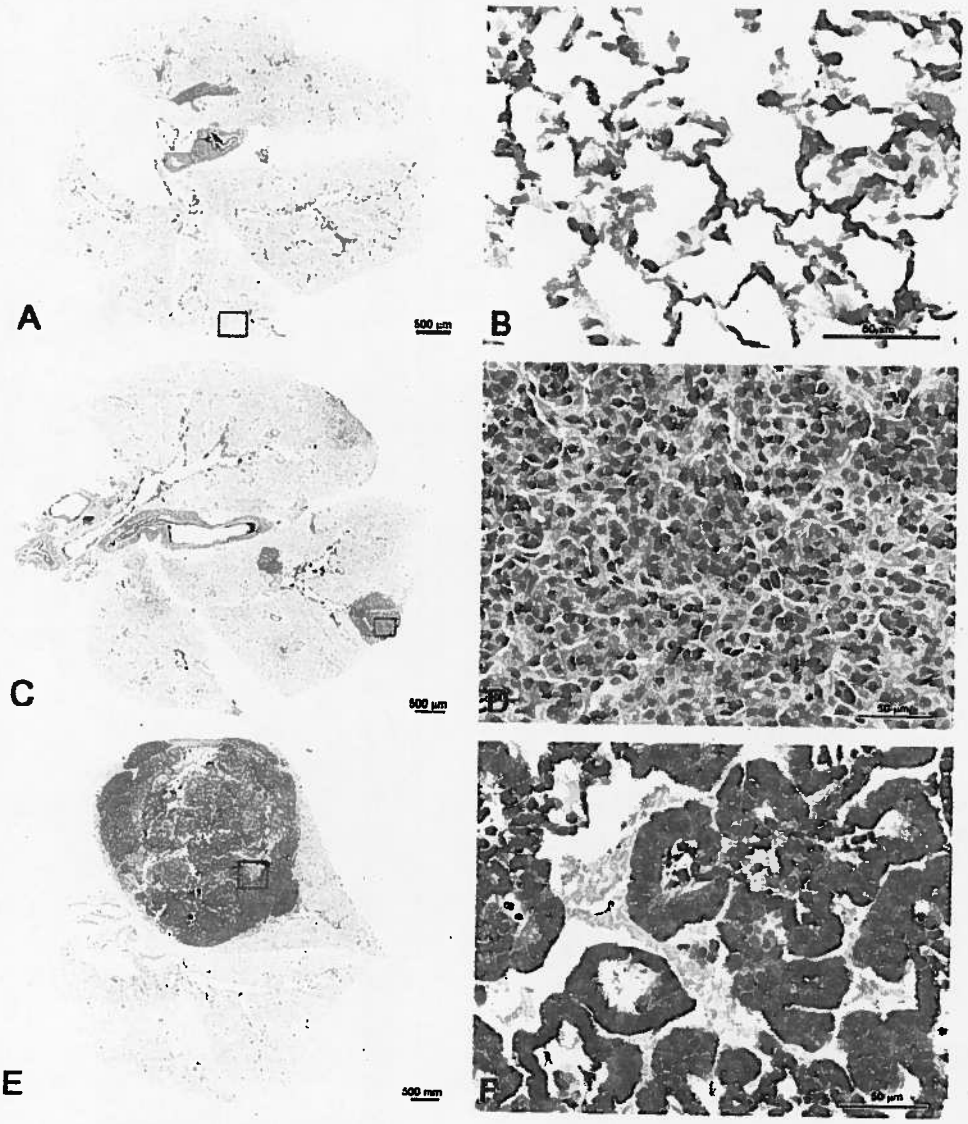


Fig. 3 Histopathological depictions of the various arsenic-induced lung bronchiolo-alveolar tumors from male mice exposed to 50 ppb as compared to a control male mouse. **a** Control lung without space occupying bronchiolo-alveolar tumors. **b** Higher magnification of area outlined in 3A showing normal alveolar acinar structures. **c** Multiple bronchiolo-alveolar adenomas consisting of circumscribed areas of tumor cells that compress, but do not invade the surrounding tissue. **d** Higher magnification of tumor cells outlined in 3C; note uniform population of cuboidal tumor cells with minimal pleomorphism and no atypia or mitosis. **e** Large bronchiolo-alveolar carcinoma nearly occupying the entire lung lobe. **f** Higher magnification of tumor cells outlined in 3E, note pleomorphic cells, ranging from low cuboidal to columnar with increased mitotic activity, and formation of papillary structures. Note this tumor histopathology is representative of typical adenomas and carcinomas seen in arsenic-treated male mice

doses which are, generally speaking, at or well above the upper range reported for human inorganic arsenic exposure from the drinking water (IARC 2004, 2012). The lung tumor response in mice seen after whole-life exposure to 24 ppm (adenoma or carcinoma in females and carcinoma in males) was not seen at the lowest dose (6 ppm; Tokar et al. 2011). Consistent with those results, in the present study, the highest arsenic dose used (5,000 ppb or 5 ppm) was not linked to lung tumors. However, in males in the present study, the lower two doses of arsenic (500 and 50 ppb) induced bronchiolo-alveolar tumors as reflected by >230 % increases in lung adenoma or carcinoma incidence. At the lowest dose (50 ppb), this increased lung tumors included a significant increase in lung carcinoma and numerically the most multiple primary lung tumors and alveolar hyperplasia. These results were not expected, particularly the unusual dose–response. The lowest whole-life dose (50 ppb) used is only five times the USEPA maximum contaminant level in drinking water for arsenic in the US (10 ppb), and a response at this level is cause for serious concern.

Table 4 Bronchiolo-alveolar tumors induced by whole-life arsenic exposure in females

Lesion	Tumor-bearing mice/total examined	Percent (%)	*p* value
Adenoma			
Control	4/38	11	–
50 ppb	9/38	25	0.043*
500 ppb	8/39	21	0.143
5,000 ppb	4/37	11	0.473
Trend *p*			0.136
Carcinoma			
Control	2/38	5	–
50 ppb	2/38	5	0.492
500 ppb	3/39	8	0.346
5,000 ppb	2/37	5	0.490
Trend *p*			0.463
Adenoma or carcinoma			
Control	6/38	16	–
50 ppb	11/38	29	0.066
500 ppb	11/39	28	0.122
5,000 ppb	6/37	16	0.481
Trend *p*			0.149
Alveolar hyperplasia			
Control	2/38	5	–
50 ppb	0/38	0	–
500 ppb	4/39	5	–
5,000 ppb	1/37	3	–

Arsenic was given continuously in the drinking water throughout life. Sample size is the number of mice available for pathological analysis. Data are expressed as mice with a given lesion (% pathologically assessed). Groups were derived from 10 to 11 litters with original $n = 40$ individuals (see Table 1 for details)

* Significant difference from control at $p < 0.05$. Data analyses are adjusted for survival and litter effects. A mouse in the 5,000-ppb treatment group had a multiple adenoma

Nonetheless, these results should be interpreted with great care. For one, the reason for the absence of a typical dose–response for lung tumor formation is unknown and requires thoughtful scrutiny and confirmation in further study.

As a toxicant, arsenic has such a multitude of diverse biological actions, and evidence indicates inorganic arsenic can show atypical dose–response effects. For instance, complex dose–response effects occur in vitro for inorganic arsenic and glucocorticoid receptor-mediated transcription such that low arsenic concentrations (<0.7 μM) stimulate transcription but slightly higher non-toxic concentrations inhibit transcription (Bodwell et al. 2004). Complex arsenic dose–response curves are shared by the progesterone and mineralocorticoid receptors (Bodwell et al. 2006). Similarly, a clear dichotomy exists in that inorganic arsenic was widely used as a cancer chemotherapeutic (Bentley and Chasteen 2002; Chen et al. 2011) long before it was ever considered an environmental carcinogen. Arsenic has been reintroduced as a cancer chemotherapeutic and is considered a "magic bullet" against certain leukemias (Chen et al. 2011) and may be useful against other cancers (Kritharis et al. 2013). However, it is clear that inorganic arsenic can both kill tumors and stimulate tumor growth depending on the dose (Soucy et al. 2003; Kamat et al. 2005; Liu et al. 2006). In human xenograft tumor model systems, oral or injected inorganic arsenic in mice clearly can kill tumor cells and block tumor growth (e.g., Liu et al. 2006), but at lower doses, it will actually stimulate tumor growth and metastasis (Soucy et al. 2003; Kamat et al. 2005; Liu et al. 2006), and often the lower the dose of arsenic, the more effective it is in this stimulation (Soucy et al. 2003; Liu et al. 2006). Indeed, some find xenograft tumor growth and metastasis is stimulated by oral doses of inorganic arsenic as low as 10 ppb (Kamat et al. 2005), and others caution that low-dose metronomic arsenic chemotherapy should be used with "extreme caution" because of carcinogenic potential (Liu et al. 2006). The current whole-life exposure study showed no increase in lung tumors at the highest dose (5,000 ppb), and one might speculate that this level acted as a "pharmacological" dose for lung cancer. The net effects of a complex, dual action carcinogen/cancer chemotherapeutic agent likely result from an intricate interplay of various factors such as target site dosimetry and length and timing of exposure. Beyond this, the mechanism of arsenic-induced lung cancer is not defined, and it is not known whether the mechanism for lung tumor induction in the moderate ppm range seen in prior whole-life work (Tokar et al. 2011) is the same as in the ppb range in the present study. Regardless, it may be that at certain doses chronic arsenic exposure initiates cancer and then acts as a chemotherapeutic for the very cancers that were initiated. In essence, arsenic could target the same cells it had earlier transformed, with the tumor outcome dependent on the dose and window of exposure. Further study is clearly required to help define the complexities of the dose–response in lung tumor formation seen in the present study.

Lung tumors in mice are often a late life event and have a propensity to be observed in older mice (e.g., Wang et al. 2012). Survival therefore may impact their formation. However, there were no significant differences in survival in the arsenic-treated males compared to control males in the present study. In females, the group exposed to 50 ppb arsenic showed reduced survival for unknown reasons but, nonetheless, showed an increase in lung adenoma formation compared to control. In addition, survival was adjusted for in all statistical comparisons of tumor incidence. Thus, the increased lung tumors associated with arsenic exposure cannot be explained by differences in survival in the present study.

In the present work, whole-life arsenic exposure in the ppb range increased lung adenoma or carcinoma in male CD1 mice but appeared less effective in females, although females did show an increase in lung adenoma at 50 ppb. Various aspects of arsenic toxicity can have significant gender differences. For example, men appear more sensitive to arsenic-induced skin effects than women, and there can be gender-related differences in inorganic arsenic methylation (Vahter et al. 2007). Further, gender differences can occur in experimental carcinogenesis studies with inorganic arsenic, although females are not always less sensitive (Tokar et al. 2010; Vahter et al. 2007). In humans, males and females often are equally susceptible to lung cancers after inorganic arsenic exposure (IARC 2004, 2012), although some data indicate higher sensitivity to lung cancer in women after arsenic exposure (Aballay et al. 2012). In the lung, in utero exposure via maternal drinking water to 10 or 100 ppb results in impaired pulmonary mechanics in the offspring during infancy in mice to a greater extent in male offspring than female (Ramsey et al. 2013a). Thus, although we have no ready explanation for the observed gender relationship in lung tumors in this study, gender differences for inorganic arsenic are not uncommon but can be complex.

In summary, in male CD1 mice, lung tumors were associated with whole-life inorganic arsenic exposure in the ppb range, a level of exposure of concern to humans (IARC 2004, 2012). This response was lost at highest dose in the low ppm range for reasons that are not completely apparent. Further work should be performed in assessing the carcinogenic potential of inorganic arsenic in mice at human relevant doses. Such a study could include aspects such as more doses in the human relevant range, larger group sizes for even greater statistical power, and more extensive pathological analyses. We are hopeful that the present work will stimulate others to investigate the carcinogenic potential, and eventually the carcinogenic mechanisms, of human relevant inorganic arsenic doses in mice.

Acknowledgments The authors wish to thank Dr. Bhalchandra Diwan, NCI-Frederick for aid with experimental design, Drs. Thayer, Sills and Bucher, DNTP, for critical evaluation of this manuscript, Dan Logsdon and Mark Schrader NCI-Frederick for expert technical assistance in animal husbandry, for provision of critical information on diet and water, and provision of diet samples, and Matthew Bell, DNTP, for assistance with graphics. This research was supported in part by the DNTP, NIEHS and in part by the National Cancer Institute, Center for Cancer Research.

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TOXICOLOGICAL SCIENCES 137(1), 36–46 2014
doi:10.1093/toxsci/kft227
Advance Access publication October 4, 2013

Characterization of Intracellular Inclusions in the Urothelium of Mice Exposed to Inorganic Arsenic

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Received August 14, 2013; accepted September 18, 2013

Inorganic arsenic (iAs) is a known human carcinogen at high exposures, increasing the incidences of urinary bladder, skin, and lung cancers. In most mammalian species, ingested iAs is excreted mainly through urine primarily as dimethylarsinic acid (DMA^V). In wild-type (WT) mice, iAs, DMA^V, and dimethylarsinous acid (DMA^{III}) exposures induce formation of intramitochondrial urothelial inclusions. Arsenite (iAs^{III}) also induced intranuclear inclusions in arsenic (+3 oxidation state) methyltransferase knockout (As3mt KO) mice. The arsenic-induced formation of inclusions in the mouse urothelium was dose and time dependent. The inclusions do not occur in iAs-treated rats and do not appear to be related to arsenic-induced urothelial cytotoxicity. Similar inclusions in exfoliated urothelial cells from humans exposed to iAs have been incorrectly identified as micronuclei. We have characterized the urothelial inclusions using transmission electron microscopy (TEM), DNA-specific 4′,6-diamidino-2-phenylindole (DAPI), and non-DNA-specific Giemsa staining and determined the arsenical content. The mouse inclusions stained with Giemsa but not with the DAPI stain. Analysis of urothelial mitochondrial- and nuclear-enriched fractions isolated from WT (C57BL/6) and As3mt KO mice exposed to arsenate (iAs^V) for 4 weeks showed higher levels of iAs^V in the treated groups. iAs^{III} was the major arsenical present in the enriched nuclear fraction from iAs^V-treated As3mt KO mice. In conclusion, the urothelial cell inclusions induced by arsenicals appear to serve as a detoxifying sequestration mechanism similar to other metals, and they do not represent micronuclei.

Key Words: **micronuclei; granules; carcinogenesis; genotoxicity; intramitochondrial; intranuclear.**

Arsenic is a metalloid element with atomic number 33. Chronic exposure to high doses of inorganic arsenic (iAs) (arsenate [iAs^V] and arsenite [iAs^{III}]) causes cancer and noncancerous effects in humans (IARC, 2004; National Research Council, 2001). iAs has been classified as a group I carcinogen since 1989 (IARC, 2004). Chronic ingestion occurs mainly through drinking water contaminated with iAs either from geologic formations or from human activity, but also occurs from food and by inhalation. Chronic high exposures to iAs ingestion leading to increased rates of urinary bladder, skin, and lung cancer are a significant public health problem in several countries including Taiwan, Mongolia, Bangladesh, India, China, Chile, Argentina, and Mexico. Certain parts of the United States have relatively high levels of arsenic in drinking water, but not as high as in those other countries (Gronberg, 2011).

In most organisms, iAs is metabolized by an alternating series of reductions of pentavalent forms to trivalent forms followed by sequential oxidative methylation. This metabolism yields iAs^{III}, monomethylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), and trimethylarsine oxide (TMA^VO) (Le *et al.*, 2000; Vahter, 2002). DMA^V is the most abundant metabolite excreted in urine of humans and rodents. Among these arsenicals, trivalent arsenicals have been shown *in vitro* to be highly reactive and considerably more cytotoxic compared with pentavalent forms, especially for the methylated arsenicals (Cohen *et al.*, 2006). These trivalent forms are believed to play a critical role in iAs-induced toxicity and carcinogenicity (Cohen *et al.*, 2007; Dodmane *et al.*, 2013; Hughes *et al.*, 2011).

As previously reported (Arnold *et al.*, 2006; Dodmane *et al.*, 2013; Suzuki *et al.*, 2008b; Yokohira *et al.*, 2010, 2011), intracellular eosinophilic inclusions are observed in the superficial layer of the urothelium of mice exposed to iAs^{III}, iAs^V, DMA^{III}, or DMA^V. These inclusions were present in all layers of the urothelium in iAs^{III}-treated arsenic (+3 oxidation state) methyltransferase knockout (As3mt KO) mice, which are unable to

methylate iAs. The inclusions were located in mitochondria and nuclei in As3mt KO mice and in mitochondria in wild-type (WT) mice. These inclusions do not appear to play a role in the urothelial toxicity of arsenicals because they do not occur in rats treated with arsenicals even though urothelial toxicity occurs in both rats and mice. It is possible that they act as a protective sequestering mechanism as seen with other metals (Brown *et al.*, 1985; Masci & Bongarzone, 1995; Gonick, 2011).

In humans, the presence of cytoplasmic inclusions in exfoliated urothelial cells in urine was reported in epidemiological studies in populations exposed to high levels of arsenic in drinking water (Basu *et al.*, 2002; Ghosh *et al.*, 2008). These inclusions have been labeled as micronuclei, often based on staining with the nonspecific Giemsa stain (Basu *et al.*, 2002) or staining with centromere-specific stains in which the majority of the inclusions were centromere negative (Ghosh *et al.*, 2008; Marchiset-Ferlay *et al.*, 2012; Moore *et al.*, 1997). We have recently reported the presence of intracytoplasmic eosinophilic inclusions, similar to those in mice, in exfoliated cells in urine from promyelocytic leukemia (PML) patients treated with arsenic trioxide (ATO) (Wedel *et al.*, 2013). These inclusions in the urothelial cells from ATO-treated patients do not stain with the DNA-specific stain 4′,6-diamidino-2-phenylindole (DAPI), indicating that the inclusions are not micronuclei, despite their similar morphology to the inclusions claimed to be micronuclei by other researchers. Furthermore, observations by transmission electron microscopy (TEM) showed that the inclusions in mice or humans were not morphologically related to micronuclei (Suzuki *et al.*, 2008a; Wedel *et al.*, 2013).

The composition of the inclusions in the mouse urothelium is not known. In our previous study (Suzuki *et al.*, 2008a), iAs^{III} was the major species identified in the mitochondrial fraction isolated from urothelial cells that were collected from the iAs^{III}-treated mice, suggesting that the inclusions were a storage depot for arsenic.

The urothelial inclusions found in arsenical-treated mice were characterized using TEM, DNA-specific (DAPI) and non-DNA-specific (Giemsa) stains. Speciation and quantitation of the arsenical content of enriched urothelial mitochondrial and nuclear fractions isolated from iAs^{V}-treated WT and As3mt KO mice were determined.

MATERIALS AND METHODS

Chemicals

Sodium arsenite ($NaAsO_2$), ≥ 99% pure, and sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), 99.7% pure, were purchased from Sigma-Aldrich (St Louis, Missouri). Focus SubCell (G-Biosciences, St Louis, Missouri) was used to isolate and enrich subcellular fractions.

Animals

WT C57BL/6 mice were purchased from Charles River Breeding Laboratories (Raleigh, North Carolina, for Experiment 1; Portage, Michigan, for Experiment 3). The As3mt KO mice were bred from 4 female and 2 male mice homozygous for the disrupted As3mt gene (Drobna *et al.*, 2009) obtained from Dr David Thomas (U.S. Environmental Protection Agency, Research Triangle Park, North Carolina). Exons 3 through 5 were deleted by homologous recombination to generate the As3mt KO homozygous mice. The altered gene was introduced and maintained in strain 129S6 mice before being bred into the C57BL/6 strain of mice by marker-assisted accelerated backcrossing performed by Charles River Laboratories (Wilmington, Massachusetts) to produce homozygous $As3mt^{-/-}$ mice. The mice were fertile, so brother/sister matings were used to maintain the homozygous As3mt KO genotype.

All animals were placed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and quarantined for at least 7 days prior to treatment. The level of care provided met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Experimental protocols were approved by the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC). Animal husbandry included housing in polycarbonate cages (5 per cage) with micro-isolator tops and dry corncob bedding. Nestlets (Ancare, Bellmore, New York) were placed inside the cages for environmental enrichment. Animals were maintained at approximately 22°C and 50% relative humidity with a 12-h light/dark schedule and were provided with pelleted Purina 5002 diet (Dyets Inc., Bethlehem, Pennsylvania) and hyperchlorinated, reverse osmosis water or tap water *ad libitum* throughout the study. Total arsenic content of the drinking water was < 8 ppb according to analyses by the Omaha Metropolitan Utilities District, and the diet had < 0.2 ppm total arsenic according to analyses provided by Purina.

Experiment 1

To evaluate iAs^{V}-induced urothelial inclusions and to determine arsenic speciation and quantification in enriched mitochondrial and nuclear fractions, 80 female WT and 80 female As3mt KO mice approximately 10–12 weeks of age were divided randomly by a weight stratification method (Martin *et al.*, 1984) into 2 groups of 40 mice each per genotype. One group in each genotype was administered 104 ppm sodium arsenate (equivalent to 25 ppm elemental arsenic) in drinking water for 4 weeks by injecting freshly prepared sodium arsenate into water bags (Hydropac, Lab Products, Seaford, Delaware) twice per week. After 4 weeks, 30 animals from each group were sacrificed, and urothelial cells were removed mechanically by scraping and stored in PBS at −80°C. The remaining 10 animals were used to evaluate urothelial changes as reported earlier (Cohen *et al.*, 2007).

Sacrifice and tissue processing. All animals were sacrificed by an overdose of Nembutal (150 mg/kg body weight, IP) followed by exsanguination after removal of specific tissues. Urinary bladders used for collection of urothelial cells were inflated *in situ* while the mice were still alive and under deep anesthesia with cold PBS. After removal, the bladders were inverted on wood applicator sticks and the epithelia scraped gently with the edge of a scalpel blade. The urothelial cells were removed by swirling the scalpel blade in microcentrifuge tubes containing PBS with protease inhibitor (Sigma) and kept at approximately −80°C until processed for purification of enriched mitochondrial and nuclear fractions. To determine efficiency of removal of the urothelium, the remaining bladder tissue was fixed in Bouin's fixative, processed for paraffin embedding, stained with H&E, and examined by light microscopy. Urinary bladders used for paraffin embedding were inflated and fixed with Bouin's fixative, along with a piece of small intestine. The paraffin-embedded tissue sections were stained with various stains described below.

Enrichment of mitochondria and nuclei. Mitochondrial- and nuclear-enriched fractions were isolated using a modification of the method developed by Nagata *et al.* (2010). Briefly, urothelial cells collected from each group in Experiment 1 were thawed and passed through a 35-μm cell strainer (BD Biosciences) to lyse the cells. The whole cell lysate was centrifuged at 3000 rpm (Eppendorf 5402) for 5 min to separate nuclei from the cytoplasmic

fraction. The crude nuclear fraction was further purified using a sucrose gradient (1.12 M sucrose, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and centrifuged for 30 min at 14000 rpm in an Eppendorf 5402 centrifuge (Eppendorf, Hauppauge, New York). The sediment was further washed in sucrose solution (250 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The cytoplasmic fraction was centrifuged at 14 000 rpm for 10 min. The sediment was retained, washed in sucrose buffer (1 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and dissolved in Mitochondrial Storage Buffer (G-Biosciences). A portion of each enriched fraction was fixed in glutaraldehyde/paraformaldehyde solution and processed for TEM examination. The remaining portions of each fraction were frozen at –80°C until processing for quantitation and speciation of arsenicals.

Quantitation and speciation of arsenicals. The frozen samples of enriched mitochondria and nuclei were thawed and centrifuged at 4000 rpm for 10 min at 4°C (Micromax RF, Thermo IEC). The supernatant was filtered through a 0.45-µm nylon membrane and diluted with deionized water for analysis of small molecule arsenic species. For analysis of all arsenic species including macromolecular-bound species, the precipitate was mixed with RIPA solution (5 mM Tris; pH 7.5, 15 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate, and 0.01% SDS), ethanol, and dimethyl sulfoxide and sonicated for 30 min followed by dilution with deionized water. The solution was centrifuged for 10 min at 4000 rpm, and the supernatant was analyzed.

Quantitative determination of the small molecule arsenic species and macromolecular-bound arsenic species was conducted by HPLC separation coupled with ICPMS detection system. For separation of the small molecule arsenic species, the methods have been described previously (Le *et al.*, 2000; Lu *et al.*, 2003; Yuan *et al.*, 2008). For separation of the macromolecular-bound arsenic species, a size exclusion column (Biosep-S 2000, 300×4.6 mm, Phenomenex, Torrence, California) was used. The mobile phase contained 35 mM NH_4HCO_3, 5% methanol, pH 8.5. The flow rate was 1.0 ml/min. The effluent from the post column was brought directly into the ICPMS (Agilent 7500 ce) for arsenic species detection. *m/z* 75 was monitored for arsenic species. Helium mode was used for interference elimination.

Because protein content of each enriched fraction was not known, the percent contribution of arsenic species to the total arsenic content of each enriched fraction was calculated to compare between the treated groups (Table 1).

Experiment 2

To evaluate inclusions in As3mt KO mice by TEM, 6 female and 4 male As3mt KO mice, approximately 31–32 weeks old, were transferred from the breeding colony and randomized (Martin *et al.*, 1984) based on sex and weight into 2 groups of 3 female mice each and 2 male mice. One group for each sex was administered 43.3 ppm sodium arsenite (25 ppm elemental arsenic) in tap water for 2 weeks. Animals were sacrificed as described in Experiment 1 except that a glutaraldehyde/paraformaldehyde fixative was used for *in situ* inflation of the urinary bladder while mice were still alive and under deep anesthesia. Bladders were removed and placed in the same fixative. The bladders were processed for examination by TEM as described previously (Suzuki *et al.*, 2008a).

Experiment 3

To evaluate urothelial recovery from inclusions after treatment and to prepare cytospin smears from urothelial scrapings, 20 female WT mice, approximately 7 weeks of age, were randomized (Martin *et al.*, 1984) by weight into 2 groups of 10 animals each and treated with 0 ppm (control) or 173.2 ppm of sodium arsenite (100 ppm elemental arsenic) for 4 weeks. After 4 weeks, 5 mice in each group were sacrificed to assess urothelial changes by histopathology (Cohen *et al.*, 2007) as described in Experiment 1. Except in 1 animal from the treated group, the urothelium was scraped as described in Experiment 1, and a single cell solution was prepared by passing the scraped material through a 35-µm cell strainer (BD Biosciences, Durham, North Carolina). A cytospin smear was prepared from this single cell solution, stained with Wright-Giemsa stain (referred to as Giemsa stain), and observed by light microscopy. Remaining mice were allowed to recover by administering regular water for an additional 90 days before they were sacrificed to assess the urothelial changes by histopathology as described in Experiment 1.

Staining Procedures

Paraffin-embedded bladder tissue was stained with H&E, Modified Wright-Giemsa Stain (Sigma-Aldrich), and DAPI as per the standard operating procedure in the Tissue Sciences Facility, UNMC. The H&E staining was carried out according to standard operating procedure developed for the autostainer using Hematoxylin 560MX and Eosin Y515 from Leica (Richmand, Illinois). Briefly, for Giemsa staining, the tissue sections were dehydrated in alcohol, dewaxed in xylene, fixed in methanol, stained with Modified Wright-Giemsa solution for 5 min, and washed. For DAPI staining, dewaxed tissue sections were dipped in DAPI solution (Sigma-Aldrich) for 5 min, washed in water, and air dried. The cytospin slide prepared from mouse urothelial scrapings using a Shandon Cytospin 3 centrifuge (Thermo Scientific, Kalamazoo, Michigan) was stained with Wright-Giemsa stain (Sigma-Aldrich) according to the standard operating procedure followed in the Department of Human Genetics Laboratory, UNMC.

TABLE 1

Determination of Arsenic Molecular Forms and Quantification in Enriched Fractions of Mitochondria and Nuclei Isolated From Urothelium of Mouse (WT or As3mt KO) Administered 0 or 25 ppm iAs^V (104 ppm sodium arsenate) in Drinking Water

			Concentration of Arsenicals, µM (% of Total Arsenic)				
Enriched Fraction	Treatment	Strain	As^V	As^{III}	MMA	DMA	Total Arsenic (µM)
Mitochondria	Control	WT	0.08 (100)	nd	nd	nd	0.08
	25 ppm As^V	WT	0.16 (92)	nd	nd	0.01 (8)	0.17
	Control	KO	0.08 (100)	nd	nd	nd	0.08
	25 ppm As^V	KO	0.15 (76)	0.04 (18)	nd	0.01 (6)	0.20
Nuclei	Control	WT	0.06 (80)	nd	0.001 (1.6)	0.02 (19)	0.08
	25 ppm As^V	WT	0.12 (54)	0.014 (6)	0.003 (1.5)	0.09 (38)	0.23
	Control	KO	0.07 (65)	0.004 (4)	0.002 (1.9)	0.03 (29)	0.11
	25 ppm As^V	KO	0.16 (28)	0.349 (59)	0.003 (0.5)	0.07 (12)	0.59

Note. Detection limit: 0.1 µg/l for all species. Abbreviation: nd, not detectable.

RESULTS

Characteristics of Inclusions in Mouse Urothelium

Repeated oral exposure of WT female mice to iAs^{V} resulted in formation of intracytoplasmic inclusions in the superficial layer of the urothelium. By H&E staining, the intracytoplasmic inclusions were eosinophilic and round, of varying sizes, with multiple inclusions frequently present per cell (Fig. 1). In contrast to the WT mice (Fig. 1B), intracytoplasmic inclusions were present in all layers of the urothelium (Figs. 1C and 2B) and in nuclei (Figs. 1C and 2D) of As3mt KO mice treated with iAs^{V} or iAs^{III}. When WT and As3mt KO mice were treated with equivalent doses of iAs^{V} (Figs. 1B and C), the number of inclusions was higher in the As3mt KO mice compared with WT mice.

Examination of Mouse Urothelial Inclusions by TEM

TEM examination of intact urothelial sections of iAs^{III}-treated mice clearly showed the presence of homogenous, round inclusions in the mitochondria (Figs. 2B and C) and irregular shaped but homogenous intranuclear inclusions in the female As3mt KO mice (Figs. 2B and D). Male As3mt KO mice did not show intranuclear inclusions. The intranuclear inclusions were not membrane bound and were highly irregular in shape, but of the same electron density as those seen in the cytoplasmic organelles. Some of the intracytoplasmic inclusions appeared to be present in lysosomes in addition to the mitochondria, which might indicate degradation of inclusion-filled mitochondria with uptake into secondary lysosomes (Fig. 2E), often showing ultrastructural features similar to what is found in autophagosomes and autolysosomes. All cytoplasmic inclusions were within membrane-bound structures; no electron-dense inclusions were identified lying free within the cytoplasm. Similar findings were observed in enriched subcellular fractions from iAs^{V}-treated As3mt KO mice (Fig. 3). In the enriched subcellular fraction from WT mice treated with iAs^{V}, inclusions were not present in nuclei. It was noted that female mice exhibited larger numbers and larger sized inclusions than male mice.

Quantitative Speciation of Arsenic Composition of Mouse Urothelial Inclusions

Chemical analysis of the enriched fractions of mitochondria and nuclei from mice treated with iAs^{V} showed that the majority of the arsenic is free and not bound to macromolecules.

iAs^{V}, which was administered to the WT and As3mt KO mice, was the predominant form of unbound arsenical found in the mitochondrial and nuclear fractions from the WT mice and in the mitochondrial fraction from the As3mt KO mice (Table 1). iAs^{III} contributed to total arsenic content (18%) only in the mitochondrial fraction from treated As3mt KO mice. iAs^{III} was not detectable in this fraction from treated WT mice. DMA was detected in mitochondrial and nuclear fractions from both treated WT (8%) and treated As3mt KO mice (6%), but it contributed less to the total arsenic than did iAs. The As3mt null genotype appears to increase accumulation of iAs^{III} compared with other arsenic species.

In the enriched nuclear fraction from the treated WT group, iAs^{V} (54%) and DMA (38%) were the major contributors to the total arsenic measured. In contrast, iAs^{III} (59%) was the major arsenic species followed by iAs^{V} (28%) and DMA (12%) in the enriched fraction from treated As3mt KO mice.

TMAO was not detected either in the mitochondrial or in the nuclear fractions from any of the groups. Total arsenic concentration was highest in the nuclear fraction from the As3mt KO group due to the high concentration of iAs^{III}.

Macromolecular-bound arsenic was detected only in the mitochondrial fraction from both WT (0.00053 nm) and As3mt (0.0019 nm) treated groups. The amount present in the nuclear fraction from both WT and As3mt mice was below the level of detection so that macromolecular binding of As could not be assessed. The amount of macromolecular-bound arsenic in the mitochondrial fraction was greater in the treated As3mt KO group than in the treated WT group, which correlated with the amount of iAs^{III} + DMA rather than with pentavalent iAs^{V}.

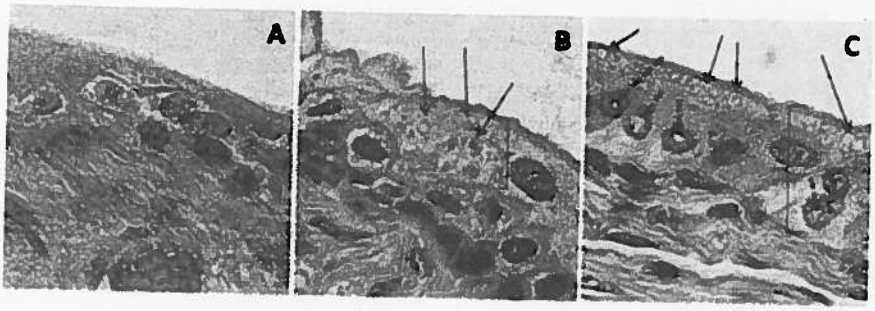

FIG. 1. By H&E staining, eosinophilic intracellular inclusions were observed in the urothelium of mice administered iAs^{V}. A, The urothelium of untreated (0 ppm As) mice. B, The presence of intracytoplasmic eosinophilic inclusions (arrows) of varying sizes in the superficial layer of the urothelium in WT C57BL/6 female mice administered 25 ppm iAs^{V} (104 ppm sodium arsenate) for 4 weeks. C, The presence of numerous intracytoplasmic (solid arrows) and intranuclear (dashed arrows) inclusions in all layers of the urothelium of As3mt KO female mice administered 25 ppm iAs^{V} for 4 weeks. ×1000. Abbreviations: As3mt KO, arsenic (+3 oxidation state) methyltransferase knockout; H&E, hematoxylin & eosin; iAs^{V}, arsenate; WT, wild type.

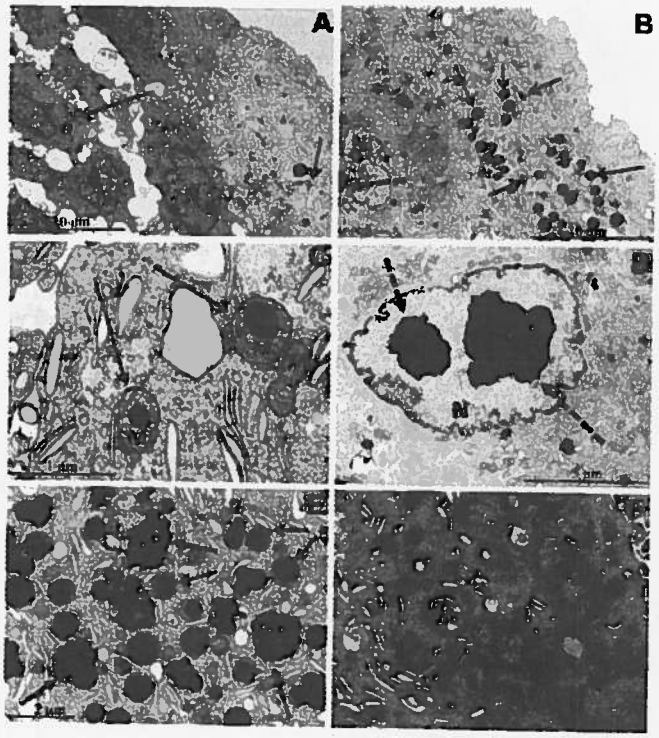

FIG. 2. Ultrastructure of inclusions in intact urothelium of urinary bladders from As3mt KO female (A–D) and male (E and F) mice exposed to 0 As (0 ppm iAs^{III}) (A) or 25 ppm iAs^{III} (43.3 ppm sodium arsenite) (B–F) for 2 weeks. There were numerous intracytoplasmic inclusions that were intramitochondrial (A–C, solid arrow), intranuclear (B and D, dashed arrow), and in secondary lysosomes (E, arrow with solid arrowhead). The male mice had myeloid bodies (My), some having inclusions (F, solid arrow with open arrowhead). Such myeloid bodies are probably related to aging but could also be related to oxidized lipid membranes. The intramitochondrial inclusions were round; intranuclear inclusions were irregular. Abbreviations: As3mt KO, arsenic (+3 oxidation state) methyltransferase knockout; iAs^{III}, arsenite.

Effect of Dose and Time of Exposure on Formation of Inclusions in Mouse Urothelium

Review of the slides from previous studies (Yokohira *et al.*, 2011) was performed by a coauthor (SMC) specifically to semiquantify and characterize the location of inclusions. Inclusion formation was found to be related directly to the amount of arsenic exposure. There was a dose-responsive relationship in the increase in the number of inclusions per mouse and in the number of mice (WT and As3mt) with inclusions (Supplementary Table S1). The number of inclusions was greater in As3mt KO compared with WT mice, and the location of the inclusions in the As3mt KO mice depended on the dose. At low doses (< 10 ppm), inclusions were intracytoplasmic and present only in the superficial layer of the urothelium. As the dose increased (≥ 10 ppm), inclusions were observed in cells in all layers of the urothelium (superficial, intermediate, and basal), and both intracytoplasmic and nuclear inclusions were observed.

The WT mice exposed to 100 ppm iAs^{III} for 4 weeks and allowed to recover for 90 days showed few inclusions in the urothelium by histopathological examination. In contrast, all of the superficial cells showed presence of numerous inclusions in each cell after 4 weeks of exposure to iAs^{III} (Fig. 4I).

Inclusion Formation in Untreated As3mt KO Mice

Male and female As3mt KO mice maintained in the breeding colony at UNMC were exposed only to the background iAs naturally present in the drinking water at levels < 8 ppb and in the diet at levels < 0.53 ppm (Teklad 8656). A few, small intracytoplasmic inclusions were observed in the urothelium of female As3mt KO mice at 165 and 439 days of age, detected by light microscopy. No inclusions were observed in male Asm3t KO mice at these ages by light microscopy, but intramitochondrial inclusions were detectable by TEM even at the age of 82 days in both male and female As3mt KO mice.

Inclusions in Urothelium Are Not Micronuclei

Inclusions in iAs^{V}-treated WT and As3mt KO mice stained positive with Giemsa stain (Fig. 4I). However, the inclusions were negative when stained with DAPI (Figs. 4A–F; Table 2), indicating that the inclusions do not contain DNA. In combination with the electron microscopic appearance, the staining pattern indicates that the inclusions do not contain chromosomal material and are not micronuclei.

DISCUSSION

iAs is a human carcinogen (IARC, 2004; National Research Council, 2001). It is nonmutagenic (Hughes *et al.*, 2011), and accumulating evidence supports a mode of action involving cytotoxicity and regenerative proliferation (Cohen *et al.*, 2007, 2013). The presence of "micronuclei" in exfoliated urothelial cells from people exposed to high levels of iAs for long periods of time has been reported in multiple epidemiological studies (Basu *et al.*, 2002; Ghosh *et al.*, 2008; Moore *et al.*, 1997) and has been suggested as evidence for an indirect genotoxic mechanism of iAs-induced carcinogenicity (Hughes *et al.*, 2011). However, Suzuki *et al.* (2008a) reported the presence of intracytoplasmic eosinophilic inclusions (referred to as granules in earlier reports) in the urothelium of mice treated with arsenicals (iAs, DMA^{V}), which were morphologically similar in appearance to those lesions claimed to be micronuclei. Recently, inclusions similar to those in mice were observed in exfoliated urothelial cells in urine from PML patients treated with ATO (150 µg/kg body weight/day for 30 days IV) (Wedel *et al.*, 2013), and those ATO-associated inclusions appeared to be the same as those reported in epidemiology studies as micronuclei (Basu *et al.*, 2002; Ghosh *et al.*, 2008). In contrast, these inclusions were not observed in rats (Suzuki *et al.*, 2008a), and rats do not produce micronuclei in response to arsenical exposure (Wang *et al.*, 2009).

Similar inclusions in humans chronically exposed to high levels of arsenic through drinking water have been reported as

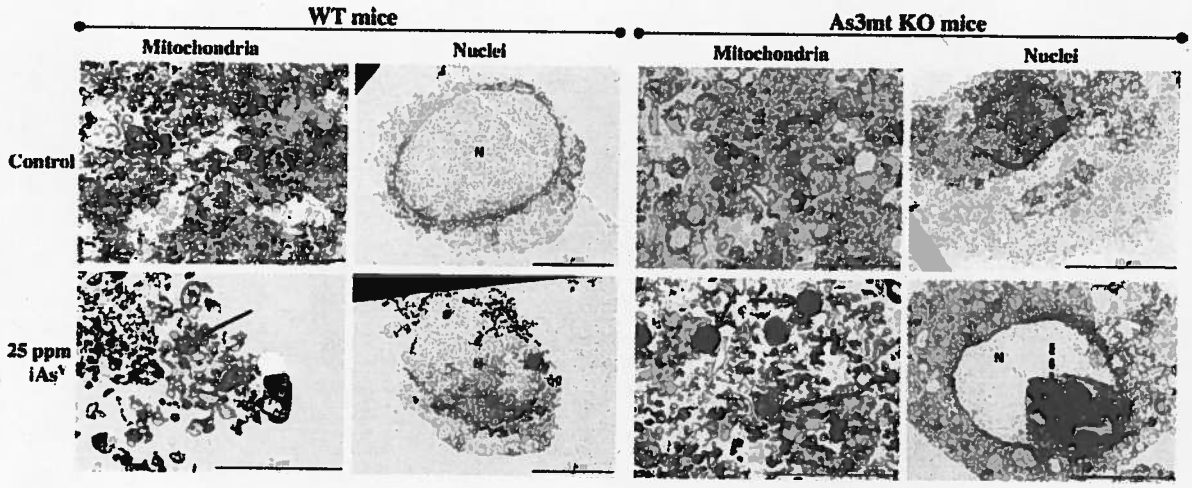

FIG. 3. Inclusions in the enriched fractions. Mice exposed to 25 ppm of iAs^V (104 ppm sodium arsenate) in drinking water for 4 weeks induced formation of inclusions in mitochondria (M, solid arrow) in both WT and As3mt KO mice and intranuclear (N) inclusions (dashed arrow) only in As3mt KO mice. Abbreviations: As3mt KO, arsenic (+3 oxidation state) methyltransferase knockout; iAs^V, arsenate; WT, wild type.

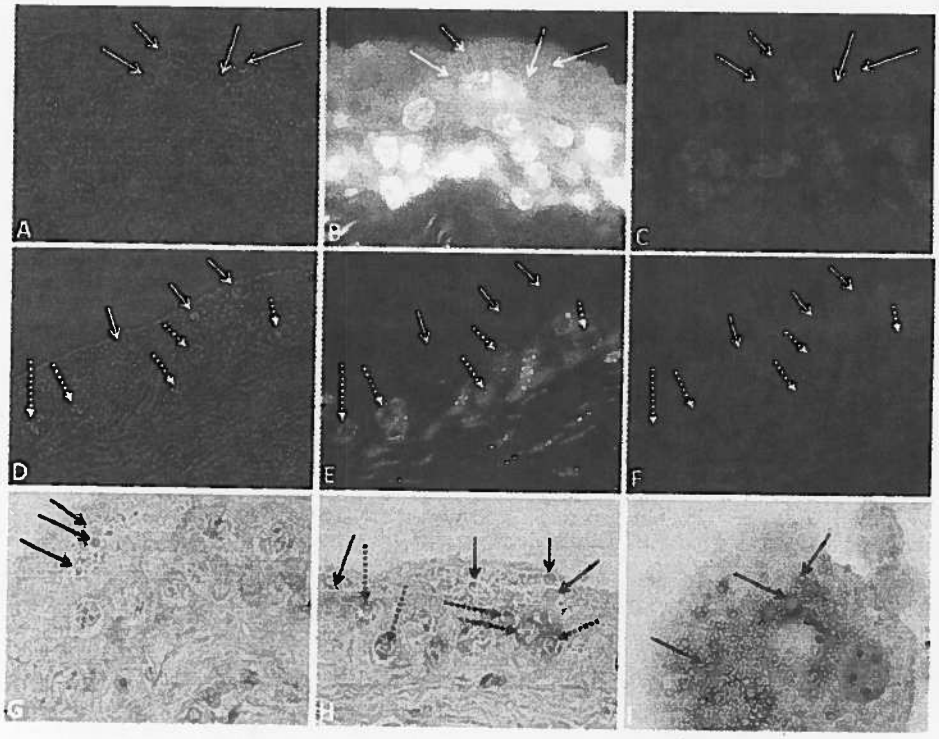

FIG. 4. The inclusions were not micronuclei. Bladder tissue sections from WT (A–C and G) and As3mt KO mice (D–F and H) exposed to 25 ppm of iAs^V (104 ppm sodium arsenate) for 4 weeks were stained with DAPI (A–F) or Modified Wright-Giemsa stain (G and H). The intracytoplasmic inclusions (solid arrows) in WT and As3mt KO mice and intranuclear inclusions in As3mt KO mice (D–F, dotted arrows) visible by light microscopy (A and D) were not visible with DAPI staining by fluorescent microscopy (B and C, E and F). By Modified Wright-Giemsa stain, the inclusions in both WT (G) and As3mt KO (H) mice stained, including the intranuclear inclusions in As3mt KO mice (H). The cytospin smear (I) of urothelial scraping from a WT mouse exposed to 100 ppm iAs^V (173.2 ppm sodium arsenite) for 4 weeks, contained numerous intracytoplasmic inclusions stained by Wright-Giemsa stain. Magnifications of pictures: A–F, ×600 and G–I, ×1000. Abbreviations: As3mt KO, arsenic (+3 oxidation state) methyltransferase knockout; iAs^{III}, arsenite; iAs^V, arsenate; DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type.

"micronuclei" (Ghosh *et al.*, 2008) using Giemsa (Basu *et al.*, 2002), Feulgen (Gonsebatt *et al.*, 1997; Tian *et al.*, 2001), or propidium iodide (Moore *et al.*, 1996) staining techniques. Feulgen staining is relatively more specific than Giemsa (Nersesyan *et al.*, 2006). However, it has been shown that lead inclusions in human and rodent tissues give a false positive reaction with the

TABLE 2

Summary of Characteristics of Intracellular Inclusions in Urothelium Induced by Exposure to Various Arsenicals in Mice and Human

				Characteristics of Inclusions						
					Location Inside Cell		Staining Characteristics			
Species	Sex	Treatment	Observed Time	Location in Urothelium	Cytoplasm	Nuclei	H&E	Giemsa	DAPI	Composition
As3mt KO mice	Female	Regular drinking water and feed iAs^{V}/[a]-[c]iAs^{III}/[d]DMA^{III}	Day 165 and day 439	Superficial layer	Yes	No	Eosinophilic			
			[b][6 and 24 h, 3, 7 and 14 days], 4 weeks	All layers (iAs), Superficial (DMA^{III})	Yes (mitochondria and lysosomes)	Yes (with iAs)	Eosinophilic	Yes	No	iAs^{V}, iAs^{III}, MMA, DMA
	Male	Regular drinking water and feed	Not observed on day 147 or 439							
		iAs^{III}	2 weeks	All layers	Yes (mitochondria and lysosomes	No				
C57BL/6WT mice	Female	iAs^{V}/[a]-[c]iAs^{III}/[d]DMA^{III}	[b][7 days, 14 days], [c]2 weeks, [d]4 weeks	Superficial layer	Yes (mitochondria)	No	Eosinophilic	Yes	No	iAs^{V}, iAs^{III}, MMA, DMA
	Male	[c]iAs^{V}, [c]iAs^{III}	2 or 10 weeks	Superficial layer	Yes	No	Eosinophilic			
B6C3F1 WT mice	Male and Female	[f]DMA^{V} in feed	2 years	Superficial layer	Yes	No				
[g]Human	[g]Female and Male	High levels of iAs in drinking water	Chronic exposure	Superficial layer	Yes	No		[g]Yes		
	Female	[h]ATO treatment for PML	0, 3, and 7 months after stopping treatment	Superficial layer	Yes (lysosomes)	No	Basophilic	Yes (data not shown)	No	

[a]Suzuki *et al.* (2008a).
[b]Yokohira *et al.* (2011).
[c]Yokohira *et al.* (2010).
[d]Dodmane *et al.*(2013).
[e]Suzuki *et al.* (2008b).
[f]Arnold *et al.* (2006).
[g]Basu *et al.* (2002).
[h]Wedel *et al.* (2013).

Feulgen stain (Beaver, 1961; Wachstein, 1949; Wolman, 1954), and therefore, they may also give a false positive when staining arsenic-induced metal ion-rich inclusions. Propidium iodide is an intercalating agent that binds DNA and RNA. In addition to its high affinity for nuclei acids (Tas and Westerneng, 1981), propidium is an inhibitor of acetylcholine esterase by binding strongly to its peripheral anionic site (Cavalli *et al.*, 2004). This suggests that propidium iodide can bind to other molecules in the cell, apart from nucleic acids. We recently reported (Wedel *et al.*, 2013) the presence of similar inclusions in exfoliated urothelial cells collected from the urine of ATO-treated PML patients. These inclusions showed negative staining for DAPI, a DNA-specific stain, and by TEM, the inclusions did not resemble micronuclei. Similarly, the inclusions in the mouse urothelial cells do not stain with DAPI and do not resemble nuclei by TEM. The intracytoplasmic inclusions are contained in membrane-bound organelles, which are not free in the cytoplasm. In studies with arsenic exposure where these "micronuclei" were categorized based on the absence or presence of centromere staining, there was an increase in centromere negative "micronuclei" compared with centromere positive "micronuclei" (Moore *et al.*, 1997). Rather than being micronuclei, they likely represent the arsenic-rich inclusions we are describing. In a few other studies, using the same method the prevalence of micronuclei was not significant compared with the unexposed group although a trend was associated with arsenic intake (Moore *et al.*, 1996; Warner *et al.*, 1994). These findings suggest that the inclusions are not micronuclei in either mice or humans, and they do not indicate genotoxicity of arsenicals.

The arsenicals iAs^{V}, iAs^{III}, and DMA^{III} all induced formation of these inclusions in the mouse urothelium in both WT (C57BL/6) and As3mt KO mice (Arnold *et al.*, 2013; Simeonova *et al.*, 2001; Suzuki *et al.*, 2008a; Yokohira *et al.*, 2010, 2011). DMA^{V}-induced inclusions were observed in B6C3F1 mice when administered through the feed (Arnold *et al.*, 2006). Inclusions in urothelial cells were also observed in the bladders of B6C3F1 mice administered iAs^{III} in the drinking water in studies conducted by the National Toxicology Program (Dr Robert Maranpot, personal communication), which were similar in appearance to what we have observed (Suzuki *et al.*, 2008a). This suggests that the induction of inclusions is not limited to one strain of mice nor is it limited to one form of administered arsenical. Based on previous studies (Arnold *et al.*, 2013; Dodmane *et al.*, 2013; Suzuki *et al.*, 2008a; Yokohira *et al.*, 2010, 2011) and the present studies, the inclusions appear similar when stained with H&E and are produced whether the arsenical is administered in the diet or in the drinking water.

In the WT mice, the inclusions were confined to the cytoplasm in the superficial cell layer of the urothelium. In As3mt KO mice, the inclusions were also present in the nuclei and in all layers of the urothelium, which is likely due to slow clearance of iAs because of lack of methylation ability (Drobna *et al.*, 2009). The presence of the inclusions in untreated mice likely corresponds to the poor ability of these As3mt KO mice to eliminate iAs (Drobna *et al.*, 2009).

Chemical analysis showed that in iAs^{V}-treated mice, WT or KO, the major arsenical in the enriched mitochondrial fractions was unbound iAs^{V}, whereas in As3mt KO mice, the major arsenical in the nuclear fraction was iAs^{III}. In a previous study, iAs^{III} was found to be the predominant arsenic species in the enriched mitochondrial fraction from WT mice administered iAs^{III} (Suzuki *et al.*, 2008a). Therefore, it appears that the major form of the arsenical present in the mitochondrial inclusions depends on the form of arsenical administered to the mice. Unlike in urine, where DMA^{V} is the major metabolite in WT mice, iAs (As^{III} + As^{V}) was the major arsenical in mitochondrial and nuclear fractions. This finding is in agreement with findings in human exfoliated urothelial cells from people exposed to high levels of iAs in the drinking water (Hernández-Zavala *et al.*, 2008) and also with our earlier report in mice treated with iAs^{III} (Suzuki *et al.*, 2008a). The arsenic species in urine do not directly correspond quantitatively to the arsenical species in the urothelial cells in mice or humans. Compared with the WT mice, As3mt KO mice appeared to store more arsenic with iAs^{V} treatment in both mitochondria and nuclei, mainly in the form of iAs^{III}, consistent with disrupted kinetics of iAs in As3mt KO mice (Drobna *et al.*, 2009).

In this study, the majority of the arsenic found in the enriched organelles was free and not bound to macromolecules. The small amount of macromolecule-bound arsenic in the enriched mitochondrial fractions coincided with amounts of As^{III} + DMA but not with iAs^{V}. This difference might be due to higher binding affinity of the trivalent iAs^{III} and DMA^{III} compared with the pentavalent iAs^{V} (Jiang *et al.*, 2003; Lu *et al.*, 2004, 2007). Unlike the mitochondrial fraction, macromolecule-bound arsenic was not detected in the enriched nuclear fractions. This might be due to weak binding of arsenic to a macromolecule or a difference in the storage buffer used for the enriched mitochondrial and nuclear fractions.

Formation of these inclusions in mice is dose (Supplementary Table S1) and time dependent (Arnold *et al.*, 2013). In As3mt KO mice, intracytoplasmic inclusions were observed as early as 6 h postexposure to drinking water containing 25 ppm of iAs^{III} compared with 7 days in the WT mice. In both WT and As3mt KO mice, exposure to iAs^{III} in drinking water for 4 weeks induced inclusions depending on dose; 1 ppm iAs^{III} induced fewer inclusions than 10 ppm or 25 ppm iAs^{III}. The location of the inclusions was also dose dependent in the As3mt KO mice, with intracytoplasmic inclusions present in the superficial layer of the urothelium at low doses (< 10 ppm) but in all layers of the urothelium and in the nuclei at higher doses (≥ 10 ppm). Even the background arsenic in the regular drinking water (< 8 ppb) produced a few inclusions in the breeding colony of As3mt KO mice; they were small and were found only in the superficial cells. Again, this is consistent with the kinetics for iAs in these KO mice (Drobna *et al.*, 2009).

The significance of these inclusions for the biological effects of arsenic in mice or in humans is unclear. However, the presence of the inclusions does not appear to be involved in the arsenical-induced cytotoxicity or hyperplasia of the urothelium in mice, as they were present in the urothelium of mice treated with iAs that did not show toxic effects (Suzuki *et al.*, 2008a; Yokohira *et al.*, 2010, 2011), and they were not found in rats, which show similar cytotoxic and proliferative effects to iAs exposure as mice (Arnold *et al.*, 2013). Furthermore, they were present in mice treated with DMA^V in the diet, even though that exposure did not produce cytotoxic, proliferative, or tumor effects in the mice, but they were not present in rats in which cytotoxic, proliferative, and tumor effects were induced by DMA^V fed in the diet (Arnold *et al.*, 2006). This suggests that these inclusions may act as a depot to sequester arsenicals and prevent adverse effects on cells.

Other metal ions, such as lead and cadmium, have been observed to induce intracytoplasmic and intranuclear inclusions or aggregates (Gonick, 2011; Moore and Goyer, 1974; Song *et al.*, 2008; Wachstein, 1949). As with arsenicals, such inclusions associated with other metals appear to serve to sequester the metal and do not represent a toxic effect of the metal. Many of these metal ions, including inorganic and organic arsenicals, are known to bind to metallothionein (Park *et al.*, 2001). In addition, lead has been shown to bind to specific proteins in the inclusions (Gonick, 2011). Lipofucsin was shown by light microscopy to form inclusions in the urothelial superficial layer of mice exposed to stress (Perše *et al.*, 2013). However, the inclusions induced by arsenicals were negative for immunohistochemical labeling of metallothionein, and they were negative for Fontana Masson staining, a stain for lipofucsin (data not shown). The specific macromolecules to which the arsenicals are bound in these inclusions remain unknown.

The urothelium has a very slow turnover rate in most species, from 6 months to 2 years (Jost, 1989; Khandelwal *et al.*, 2009; Rebel *et al.*, 1994; Tiltman and Friedell, 1972), unless there is cytotoxicity to the urothelium. The inclusions persisted even 7 months after the end of the arsenic treatment in human PML patients (Wedel *et al.*, 2013). We have also found that a few inclusions remain in the mice 3 months after cessation of the iAs^{III} treatment. Such persistence is consistent with the slow loss by exfoliation over time with the normal turnover of the urothelium and is consistent with the inclusions being noncytotoxic. If cytotoxic, a more rapid turnover would be expected (Cohen, 1998).

In summary, exposure to iAs induced formation of inclusions in the mouse urothelium that appear similar to the inclusions found in exfoliated urothelial cells from humans exposed to high levels of iAs. The number, extent of urothelial involvement, and time of appearance are dose dependent. The inclusions appear to serve as a depot for arsenicals and are not related to arsenical-induced urothelial toxicity. In humans, these inclusions have been mistaken for micronuclei. The current findings show that these inclusions are not micronuclei and that they are not evidence of genotoxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

Arsenic Science Task Force; Alberta Health; Alberta Innovates; the Canada Research Chairs Program; the Canadian Institutes of Health Research; the Natural Sciences and Engineering Research Council of Canada. Study design, data collection and interpretation, and this publication's contents are the sole responsibility of the authors and do not necessarily represent the official views of the funding agencies.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of the Tissue Sciences Facility and the Electron Microscopy Facility at UNMC, and we sincerely thank Dr Changhai Tian, University of Nebraska Medical Center, Omaha, NE, for providing technical advice on isolating subcellular fractions.

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Arch Toxicol
DOI 10.1007/s00204-014-1328-1

LETTER TO THE EDITOR

Re: Waalkes et al.: Lung tumors in mice induced by "whole-life" inorganic arsenic exposure at human-relevant doses, Arch Toxicol, 2014

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Received: 25 July 2014 / Accepted: 31 July 2014

The article by Waalkes et al. (2014) on lung tumors in mice induced by inorganic arsenic following "whole-life" exposure is problematic, especially when compared to these investigators' previous publications (specifically, Tokar et al. 2011).

The major difficulties with this model of Waalkes are the lack of consistency and reproducibility. In the present study, Waalkes et al. saw a statistically significant increase ($p \leq 0.05$) in lung adenomas in males only at 500 ppb inorganic arsenic, but not at 5,000 ppb, and in females, only at 50 ppb and not at higher doses. A statistically significant increase in lung carcinomas was found only in males and only at 50 ppb, not at higher doses. The authors note the absence of a "typical dose response". In this context, it is important to compare the current data with the authors' previous study (Tokar et al. 2011) in which doses of 6,000, 12,000, and 24,000 ppb inorganic arsenic did not increase adenomas in any group, and a statistically significant increase in carcinomas was seen in males only at 24,000 ppb and in females at 12,000 and 24,000 ppb.

The peculiar, conflicting results are likely due to the high variability in incidence of lung tumors in CD-1 mice. The well-known wide variability in background incidences of lung tumors in CD-1 mice needs to be incorporated into the interpretation of the results of these studies. Reference to historical controls has not been provided by Dr. Waalkes and associates for the facilities in which their studies have been performed.

Charles River Laboratories reported historical controls (Giknis and Clifford 2005) for males: adenoma incidences ranging between 2 and 42 %, and carcinomas between 1.43 and 26 %. In females, the ranges are 1.67–26.6 % for adenomas and 0.77–18.37 % for carcinomas. The variability in background incidences of these tumors in control mice is evident in the two studies reported by Waalkes' group (Tokar et al. 2011; present paper) (see attached Table 1). Comparing the incidence of adenoma plus carcinoma at 50 ppb in males to the controls of the previous study (51 vs. 34 %) utilizing a Fisher exact test (one- or two-sided) gives a p value >0.05. p is also >0.05 for a similar comparison for females. Furthermore, the incidences of adenomas and of carcinomas in all groups in Waalkes et al. (2014) are within historical controls reported by Charles River. Thus, it is difficult to make a case for them being treatment related.

In addition, the statistical criterion of significance used by Waalkes et al. does not follow the criteria of Haseman et al. (1986). When evaluating the incidence of common tumors (background incidence ≥1 %), Haseman et al. (1986) from the National Toxicology Program (NTP) recommended a p value <0.01 be utilized for statistical significance, not $p < 0.05$ as used by Waalkes. Using this criterion, the Waalkes tumor data are not significant, even using concurrent controls for comparison.

The lack of other tumors in Waalkes et al. (2014) such as liver, adrenal, and ovary, which was reported by the Waalkes group utilizing the same model (Tokar et al. 2011), is also noteworthy.

An additional complication in the present experiment was the differences in survival between groups, especially the low-dose groups being considerably less than other groups. For example, in the low-dose females only 2 of

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Published online: 12 August 2014

Table 1 Incidences of lung tumors in two "whole-life" experiments on inorganic arsenic

	Dose (ppb)[a]													
	Males	Females	M	F	M	F	M	F	M	F	M	F	M	F
	0								6,000		12,000		24,000	
Previous study[b]														
Adenomas	24	14							10	14	18	21	7	7
Carcinomas	10	7							28	21	32	28*	39*	43*
Adenomas or carcinomas	34	21							38	24	39	48*	46	50*
	0		50		500		5,000							
Current study[c]														
Adenomas	14	11	27	25*	38*	21	15	11						
Carcinomas	8	5	27*	5	19	8	15	5						
Adenomas or carcinomas	22	16	51*	29	54*	28	28	16						

* Statistically significant compared to control of respective study, $p \leq 0.05$

[a] Incidences listed as percent, as in published articles

[b] Tokar et al. (2011)

[c] Waalkes et al. (2014)

38 mice (5 %) survived to the end of the experiment. The survival differences might have impacted the difference in tumor incidences. Moreover, the short survival in all study groups raises serious questions about the acceptability of the study for evaluating carcinogenicity. The present study would not be considered acceptable based on the criteria for survival in cancer bioassay guidelines established by the NTP and regulatory agencies.

Their discussion about the dose response at the high dose in this study versus the lower doses also seems contrived. The suggestion that there is a pro-carcinogenic effect at the lower dose and anti-carcinogenic effect at the higher dose (5,000 ppb) does not fit the results of the previous study, which had increased tumors at the two highest doses (Tokar et al. 2011). Furthermore, the doses required for an anti-cancer effect in humans are considerably higher than the doses used in this experiment. For example, the treatment for promyelocytic leukemia (PML), the only tumor for which inorganic arsenic currently is therapeutically known to be efficacious, is 150 μg per kg per day, intravenously, for 30–60 days.

In the Discussion, Waalkes et al. state "...these results should be interpreted with great care. For one, the reason for the absence of a typical dose–response for lung tumor formation is unknown and requires thoughtful scrutiny and confirmation in further study." It is our opinion, based on the discussion presented above, that the results in the Waalkes et al. manuscript show no compound-related effect of As on lung tumor incidence at any dose.

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A Dose–Response Study of Arsenic Exposure and Markers of Oxidative Damage in Bangladesh

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Objective: To evaluate the dose–response relationship between arsenic (As) exposure and markers of oxidative damage in Bangladeshi adults. **Methods:** We recruited 378 participants drinking water from wells assigned to five water As exposure categories; the distribution of subjects was as follows: (1) less than 10 μg/L ($n = 76$); (2) 10 to 100 μg/L ($n = 104$); (3) 101 to 200 μg/L ($n = 86$); (4) 201 to 300 μg/L ($n = 67$); and (5) more than 300 μg/L ($n = 45$). Arsenic concentrations were measured in well water, as well as in urine and blood. Urinary 8-oxo-2′-deoxyguanosine and plasma protein carbonyls were measured to assess oxidative damage. **Results:** None of our measures of As exposure were significantly associated with protein carbonyl or 8-oxo-2′-deoxyguanosine levels. **Conclusions:** We found no evidence to support a significant relationship between long-term exposure to As-contaminated drinking water and biomarkers of oxidative damage among Bangladeshi adults.

Long-term exposure to arsenic (As)-contaminated drinking water is associated with increased risk for cancers of the skin, lungs, kidney, bladder, and liver.[1] In addition, evidence suggests As exposure increases the risk of developing cardiovascular disease.[2–5] Some research also supports a link between As and type 2 diabetes,[6–8] although this subject is controversial.[9,10] In Bangladesh alone, tens of millions of people are exposed to As-contaminated drinking water, putting them at increased risk for the attendant health problems.[11]

Although the modes of action by which As causes disease are not completely understood, an increased level of oxidative stress is widely believed to be one possible mechanism.[12] Additional potential mechanisms, many of which are not mutually exclusive, include altered DNA repair capacity,[13,14] epigenetic dysregulation,[15–17] enzyme inhibition,[18] endocrine disruption,[19,20] and chromosomal instability.[21–23] Reactive oxygen species have the potential to damage critical molecules in the cell, including DNA and proteins. Increased levels of free radicals and oxidative damage have been observed in various types of cancers,[24,25] as well as chronic conditions such as cardiovascular disease[26,27] and diabetes.[28] Thus, oxidative stress has been implicated in many of the conditions linked to As exposure, though the causal relationship between reactive oxygen species and these health outcomes remains unclear.

In vitro and in vivo studies indicate that As induces oxidative damage. For example, treatment of human–hamster hybrid fibroblast cells, rat lung epithelial cells, and breast-cancer cells with sodium arsenite at doses ranging from 2 to 15 μM has been shown to result in higher levels of reactive oxygen species and 8-oxo-2′-deoxyguanosine (8-oxo-dG), a biomarker of DNA damage.[29–31] Similarly, rats exposed to either 100 mg/L sodium arsenite or 200 to 400 mg/L dimethylarsenic acid (DMA) in drinking water display increased levels of various markers of oxidative stress, including glutathione disulfide, malondialdehyde, a marker of lipid oxidation,[32] and 8-oxo-dG.[33,34] It should be noted that the levels of As exposure used in these studies are orders of magnitude higher than those humans are exposed to via naturally contaminated drinking water.

Some, but not all, studies in humans have yielded evidence indicating that As exposure is associated with oxidative damage. Three population-based studies with sample sizes greater than 100 and detailed information about potential confounders have been performed to assess the relationship between As and DNA damage, as measured by urinary 8-oxo-dG. One reported a significant positive association between urinary As and 8-oxo-dG in 212 pregnant women in Matlab, Bangladesh.[35] The other two studies found no such association. One of these null studies was performed in Arizona and Sonora, where levels of drinking-water As were relatively low (less than 40 μg/L; $n = 124$),[36] and the other took place in the Andes of northern Argentina, where exposure was higher (median level of water As, approximately 200 μg/L; $n = 108$).[37] Finally, a recent study on the same Bangladeshi sample investigated in the present article reported that water, urinary, and blood As levels were all inversely associated with levels of blood glutathione, an important endogenous antioxidant.[38] Nevertheless, As exposure was not linked to levels of blood glutathione disulfide, the oxidized form of glutathione that increases under conditions of oxidative stress.[38] In summary, evidence supporting a causal relationship between long-term As exposure and measures of oxidative damage in humans has not yet been firmly established.

In this study, we set out to evaluate the dose–response relationship between As exposure and oxidative damage. We conducted a cross-sectional study of 378 individuals with long-term drinking water As exposure in Araihazar, Bangladesh. A wide range of water As concentrations is present in this area, and detailed information about exposure and potential confounders is available. We collected As measurements for drinking water, as well as for blood and urine from each individual in the study. Finally, we measured two biomarkers of oxidative damage: (1) plasma levels of protein carbonyls, a measure of protein damage; and (2) urinary 8-oxo-dG, to investigate damage to DNA.

From the Departments of Environmental Health Sciences (Dr Harper, Ms Ilievski, Ms Oka, Ms Calancie, Ms Slavkovich, Dr Graziano, and Dr Gamble), Biostatistics (Dr Liu and Ms Levy), and Epidemiology (Dr Hall), Mailman School of Public Health, Columbia University, New York, NY; Columbia University Arsenic Project in Bangladesh (Mr Siddique and Mr Alam), Dhaka, Bangladesh; Lamont-Doherty Earth Observatory (Dr Mey and Dr van Geen), Columbia University, Palisades, NY; and Kingsbridge Community College (Dr Mey), New York, NY.

This work was supported by grants 1 P42 ES10349, P30 ES009089, RO1 CA133595, RO1 ES017875, and K99 ES018890 from the National Institutes of Health. The Cancer Epidemiology Training Program at the Mailman School of Public Health (5-R25-CA 094061) and the Robert Wood Johnson Health & Society Scholars program also provided financial support.

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute, the National Institute of Environmental Health Sciences, or the National Institutes of Health.

The authors declare that they have no conflicts of interest.

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DOI: 10.1097/JOM.0000000000000166

METHODS

Study Overview

The study site, Araihazar, is located roughly 30 km east of Dhaka, Bangladesh. Araihazar is 1 of 509 thanas, or administrative units, in Bangladesh. In the year 2000, the Health Effects of Arsenic Longitudinal Study cohort recruitment began, eventually enrolling roughly 30,000 adults.[39] Every two years, both As exposure and the development of various health outcomes are assessed in study participants. A subset of the Health Effects of Arsenic Longitudinal Study cohort was recruited into the Folate and Oxidative Stress study between February and August of 2008, forming the basis of this investigation.

Study Sample

Study participants between the ages of 30 and 65 years were selected on the basis of their well water As concentrations, so that the final study sample represented the full range of water As values in the region. We set out to recruit 75 individuals in each of five water As exposure categories: (1) less than 10 μg/L (corresponding to the World Health Organization's drinking water standard); (2) 10 to 100 μg/L; (3) 101 to 200 μg/L; (4) 201 to 300 μg/L; and (5) more than 300 μg/L. This approach was chosen to maximize the ability to study dose–response relationships. Exclusion criteria included (1) women currently pregnant; (2) individuals taking nutritional supplements; (3) individuals who had not been drinking water from their current well for at least 3 months; and (4) individuals with diseases known to be associated with oxidative stress, such as diabetes, cardiovascular disease, or renal disease. As a result of water remediation and well-switching interventions between the year 2000 and the time of recruitment for the Folate and Oxidative Stress study, some water As categories included more or less than the 75 participants originally planned. The final study sample totaled 378 participants. Well water As concentrations were remeasured at the time of recruitment into the Folate and Oxidative Stress study. The distribution of subjects within water As categories was as follows: (1) less than 10 μg/L ($n = 76$); (2) 10 to 100 μg/L ($n = 104$); (3) 101 to 200 μg/L ($n = 86$); (4) 201 to 300 μg/L ($n = 67$); and (5) more than 300 μg/L ($n = 45$).

At each recruitment visit, a trained staff member described the purpose of the study to the participant. A Bangladeshi field staff physician read an approved assent form to each participant, after which oral informed consent was obtained and a subsequent visit to the field clinic was scheduled. At the field clinic, a trained interviewer administered a detailed questionnaire to each participant and a physician collected a venous blood sample.

This study was approved by both the Bangladesh Medical Research Council and the institutional review board at Columbia University Medical Center.

Collection of Biological Samples

Each participant came to our field clinic in Araihazar where blood and urine samples were collected and processed. Spot-collected urine samples were stored in 50-mL acid-washed tubes and frozen at −20°C. Blood was collected in EDTA-containing tubes; not all participants were fasting at the time of collection. Plasma samples were immediately separated at our field clinic, and blood and plasma samples were frozen in −80°C freezers in Araihazar. Samples were later transported in batches to Dhaka on dry ice, and stored in our Dhaka laboratory at −80°C. All samples were transported in coolers with dry ice to Columbia University, where they were analyzed.

Laboratory Assays

Water As

Our field sample collection and laboratory analysis procedures have been described elsewhere in detail.[40,41] Briefly, water samples were collected in 20-mL polyethylene scintillation vials. At least 48 hours before analysis, high-purity Optima HCl (Fisher Scientific, Pittsburg, PA) was used to acidify the samples to 1%.[42] After being diluted 1:10, water samples were analyzed using high-resolution inductively coupled plasma mass spectrometry (ICP-MS). A germanium (Ge) spike was added to correct fluctuations in instrument sensitivity. The detection limit of the method is typically less than 0.2 μg/L. The intra- and interassay coefficients of variation (CVs) for the assay were 6.0% and 3.8%, respectively.

Blood As

Whole blood As was measured using ICP-MS with a dynamic reaction cell,[43,44] with the modifications to the blood sample preparation process suggested by the Laboratory for ICP-MS Comparison Program, Institut National de Sante Publique du Quebec. Briefly, whole blood samples were thawed, mixed, and then diluted 50× with a solution containing 1% HNO_3, 1% methanol, 0.2% Triton-X-100, and 0.5% NH_4OH. Diluted samples were centrifuged for 10 minutes at 3500 revolutions per minute, and the analysis was performed on the supernatant. Matrix-induced interference was corrected using an iridium (Ir) internal standard and the instrument's dynamic reaction cell technology feature. Quality-control samples with known As concentration were obtained from the Quebec Institute and run each time the instrument was calibrated, as well as after every 10 samples. For study samples, the intra- and interprecision coefficients were 2.1% and 4.9%, respectively. Total blood As was obtained by adding inorganic As, monomethylarsonic acid, and DMA concentrations.

Urinary As

Urinary As was measured using the Aanalyst 600 graphite furnace system (PerkinElmer, Shelton, CT), as described previously.[45] The intraclass correlation coefficient between the samples calibrated by the Quebec laboratory and our laboratory's values was 0.99. Specific gravity was measured using a handheld refractometer (TS 400; Reichert, Depew, NY). In addition, urinary As metabolites were measured as previously described.[46] The method involves the separation of arsenobetaine, arsenocholine (AsC), arsenate, arsenite, monomethylarsonic acid, and DMA, using high-performance liquid chromatography, followed by detection using ICP-MS. The intra- and interassay CVs for urinary As were 3.9% and 5.6%, respectively, for quality-control samples. For study samples, the intra- and interassay CVs were 3.8% and 5.1%, respectively. Total urinary As was obtained by adding inorganic As, monomethylarsonic acid, and DMA concentrations.

8-oxo-2′-deoxyguanosine

8-oxo-2′-deoxyguanosine was measured in urine by using the "New 8-OHdG Check" enzyme-linked immunosorbent assay kit at the Genox Corporation's laboratory (Baltimore, MD). Before analysis, samples were centrifuged at 6000*g* for 5 minutes to remove particulate matter. Samples were measured in triplicate. The detection level for this assay is 0.64 ng/mL urine. The average intra- and interassay CVs were calculated using control samples run in triplicate on 21 plates; they were 7.5% and 7.7%, respectively.

Protein Carbonyls

Plasma protein carbonyls were measured using a noncompetitive enzyme-linked immunosorbent assay on the basis of the method described by Winterbourn and Buss,[47] as explained in detail in Zipprich et al.[48] Briefly, the protein concentration of each sample was

measured using a Bicinchoninic Acid Kit (Sigma-Aldrich, St Louis, MO). Proteins were derivatized using 2,4-dinitrophenylhydrazine. This was followed by the addition of a primary antibody (anti-dinitrophynyl-KLH Rabbit + immunoglobulin G 1:1500) and then a secondary antibody (streptavidin-biotinylated horseradish peroxidase complex). 3,3′,5,5′-tetramethylbenzidine was used as a visualizing reagent, after which the reaction was stopped using sulfuric acid and plates were read at 450 nm. Samples were measured in duplicate. The average intra- and interassay CVs were calculated using controls run in duplicate on 12 plates; they were 2.0% and 13.8%, respectively.

Statistical Analysis

Descriptive statistics were calculated to describe the sample characteristics, overall and by sex. To examine bivariate associations between variables, Spearman correlations were used for two continuous variables, and *t* tests were used to detect mean differences in continuous variables between two levels of binary variables, including sex. Before performing *t* tests, the appropriate transformations were applied to variables with skewed distributions.

Linear regression models were used to examine the relationship between each As exposure variable and protein carbonyls, with and without adjustment for potential confounding factors. Because 8-oxo-dG had a skewed distribution, with 18% ($n = 68$) of values lower than the detection limit of 0.64 ng/mL urine, we set lower-than-the-detection-limit values to half the detection limit, or 0.32 ng/mL urine; dichotomized the variable at its median, adjusted for urine concentration (10.9 ng/mL urine); and used logistic models to assess the association between 8-oxo-dG levels and each of the As variables, adjusting for covariates. We derived odds ratios with 95% confidence intervals from the model parameters. Candidate control variables included those suggested in the literature or our previous studies or those that showed associations with both As exposure and an outcome variable at a significance level of 0.10 in the bivariate analysis. Control variables were included in the final models if adding them resulted in a change to *B* of at least 10%. Urinary 8-oxo-dG and urinary As levels were adjusted for urine concentration by applying the following equation: [(sample mean for specific gravity − 1)/(individual specific gravity − 1)] × (individual urinary analyte value).[49] Models with and without adjustment for urine concentration were run. As the main predictors in our models, markers of As exposure were investigated both as continuous and categorical variables, divided into water groups or quintiles. Variables with skewed distributions were transformed to reduce the impact of extreme values or improve the fit of models. Blood and urinary As were transformed using the natural logarithm, and water As was square root transformed.

RESULTS

Subject Demographics

Demographic information on the subjects is presented in Table 1. The sample included a similar number of men ($n = 184$) and women ($n = 194$). The median age of our participants was 42 years, and most had only a few years of education (median duration: 4 years for men, 1 year for women). Median water As levels were 114.0 μg/L, but ranged from 0.4 to 700 μg/L. Smoking was common among men (55.6%) but not among women (2.6%), and betel nut was used equally by both sexes (approximately 35%). Fifty-eight percent of individuals in this study owned a television.

No potential confounders were associated with markers of oxidative stress at the $P < 0.05$ level. Nevertheless, we observed a trend toward an inverse correlation between years of education and plasma protein carbonyl levels (Spearman $r = -0.09$; $P = 0.07$). There was also a trend toward television owners being more likely to have urinary 8-oxo-dG values higher than the median (53.6% of owners vs 44.9% of nonowners; $\chi^2 = 2.78$; $P = 0.10$).

Plasma Protein Carbonyls

No significant associations between water, blood, or urinary As and plasma protein carbonyl levels were detected, either in bivariate analysis or in linear regression analysis, with As variables included as continuous and categorical variables. The results of linear regression analyses with As exposure variables included as continuous variables are presented in Table 2. No potential confounders were associated with both protein carbonyl and As levels. We fit the models adjusted for current smoking status and found no appreciable changes in the results. Mean protein carbonyl values for each water As exposure group are shown in Table 3.

Urinary 8-oxo-dG

No significant positive associations between As exposure and urinary 8-oxo-dG levels higher than the median were detected, whether As variables were included in the models as continuous or categorical variables. The results of logistic regression analyses with As exposure variables included as continuous variables are presented in Table 4. Among the potential confounders, only television

TABLE 1. Descriptive Statistics for Folate and Oxidative Stress Study Participants

	Total Sample ($N = 378$)*	Men ($n = 184$)*	Women ($n = 194$)*	P†
Age, yr	42 (30–63)	44 (31–63)	40 (30–62)	0.006
Education, yr	3 (0–16)	4 (0–14)	1 (0–16)	0.11
BMI, kg/m^2	19.7 (13.8–35.3)	19.0 (14.2–32.3)	20.7 (13.8–35.3)	0.003
Water As, μg/L	114.0 (0.4–700.0)	114.1 (0.4–700.0)	113.1 (0.4–492.8)	0.67
Blood As, μg/L	12.5 (2.5–53.1)	14.1 (3.7–53.1)	11.5 (2.5–37.9)	<0.001
Urinary As, μg/L‡	170.4 (12.9–1088.3)	171.2 (15.2–1015.0)	170.1 (12.9–1088.3)	<0.001
8-oxo-dG, ng/mL urine‡	10.9 (0.5–301.3)	10.9 (0.6–71.9)	11.0 (0.5–301.3)	<0.001
Protein carbonyls, ng/g proteins	243.0 (75.1–383.0)	249.2 (75.1–362.9)	235.1 (165.5–383.0)	0.39
Current smoker	28.2	55.6	2.6	<0.0001
Current betel nut user	35.8	35.0	36.5	0.77
Television owner	58.2	59.2	57.2	0.69

*Values have been given as median (range) or percentage.
†*P* values were obtained from testing for differences between the sexes, using *t* tests for continuous variables and chi-squared tests for binary variables.
‡Adjusted for specific gravity.
BMI, body mass index; 8-oxo-dG, 8-oxo-2′-deoxyguanosine.

TABLE 2. Associations Between Arsenic Exposure and Plasma Protein Carbonyls, Using Multiple Linear Regression Models*

	Full Sample ($N = 378$)	Men ($n = 184$)	Women ($n = 194$)
	B (95% CI)		
Water As, μg/L	0.17 (−0.56 to 0.90)	0.41 (−0.59 to 1.41)	−0.12 (−1.16 to 0.92)
Blood As, μg/L	3.44 (−5.85 to 12.7)	0.86 (−12.3 to 14.0)	5.22 (−8.25 to 18.7)
Urinary As,† μg/L	−0.94 (−4.65 to 2.77)	−2.57 (−8.37 to 3.22)	0.14 (−4.69 to 4.96)
Urinary As,‡ μg/L	−0.37 (−4.94 to 4.20)	−0.40 (−7.44 to 6.64)	−0.45 (−6.47 to 5.57)

*Blood As and urinary As were log transformed before inclusion in models. Water As was square root transformed.
†Not adjusted for specific gravity.
‡Adjusted for specific gravity.
CI, confidence interval.

TABLE 3. Mean Levels of Plasma Protein Carbonyls and Urinary 8-oxo-dG for Each of Five Water As Exposure Groups*

Water As Exposure Group	Plasma Protein Carbonyls, ng/g	Urinary 8-oxo-dG, ng/mL Urine
Group 1: <10 μg/L As ($n = 76$)	253.14 ± 40.43	13.09 ± 34.16
Group 2: 10–100 μg/L As ($n = 104$)	233.96 ± 42.24	10.47 ± 7.30
Group 3: 101–200 μg/L As ($n = 86$)	238.10 ± 42.66	12.80 ± 8.37
Group 4: 201–300 μg/L As ($n = 67$)	245.48 ± 43.62	11.96 ± 10.35
Group 5: >300 μg/L As ($n = 45$)	253.99 ± 44.41	10.10 ± 5.52

*8-oxo-2′-deoxyguanosine levels were adjusted for urine concentration, using specific gravity. Values are given as mean ± SD.
8-oxo-dG, 8-oxo-2′-deoxyguanosine.

TABLE 4. Odds Ratios for the Association Between Each Arsenic Exposure Measure and 8-oxo-2′-deoxyguanosine Levels Higher Than the Median, Derived From Logistic Regression Models[a]

	Full Sample ($N = 377$)	Men ($n = 184$)	Women ($n = 193$)
Urinary analytes unadjusted for specific gravity, μg/L			
Water As	1.02 (0.99–1.06)	1.02 (0.97–1.06)	1.03 (0.98–1.09)
Blood As	0.79 (0.51–1.21)	0.94 (0.52–1.71)	0.65 (0.34–1.23)
Urinary As	3.58 (2.70–4.76)*	4.04 (2.62–6.23)*	3.26 (2.25–4.75)*
Urinary analytes adjusted for specific gravity, μg/L			
Water As	1.03 (1.00–1.07)	1.02 (0.97–1.07)	1.05 (1.00–1.10)
Blood As	0.89 (0.58–1.36)	0.96 (0.53–1.74)	0.87 (0.46–1.63)
Urinary As	1.18 (0.95–1.47)	1.06 (0.77–1.47)	1.29 (0.97–1.73)

*$P < 0.0001$.
[a]Water As was transformed using the square root. Blood As and urinary As were transformed using the natural logarithm. All models were adjusted for television ownership. Values are given as odds ratio (95% confidence interval).

ownership was associated with both 8-oxo-dG and As levels and thus included in the models. When current smoking status was included in the models, no appreciable differences in the results were found. Models with and without adjustment for urine concentration gave similar results, except for the unadjusted model that included urinary As as a predictor of urinary 8-oxo-dG (Table 4). A strong association was found between these two variables ($P < 0.0001$), as would be expected because they are both strongly influenced by urine concentration. Mean 8-oxo-dG level values for each water As exposure group are shown in Table 3.

DISCUSSION

Given the premise that oxidative damage to the cell's structural molecules lies on a causal pathway leading from As exposure to associated diseases, one would expect to observe elevated levels of oxidative damage in individuals with long-term As exposure who have not yet become ill. In this cross-sectional, dose–response study of generally healthy individuals, we found no evidence of an association between water, blood, and urinary As levels and plasma protein carbonyl or 8-oxo-dG levels.

This study has a number of strengths. First, a wide range of water As levels, from 0.4 to 700 μg/L, was examined. Second, subjects were selected by exposure level to yield a relatively even distribution across a wide range of water As concentrations, an asset when studying potential dose–response relationships between exposure and markers of oxidative damage. Third, three measures of As exposure were examined (well water, blood, and urinary As levels), to optimize our assessment of exposure status. Fourth, extensive information on possible confounding variables, including age, sex, body mass index, education level, cigarette and betel nut use,

and television ownership (an indicator of socioeconomic status), was collected. Fifth, two different measures of oxidative damage were examined, one targeting damage to proteins (plasma protein carbonyls) and the other to DNA (urinary 8-oxo-dG). These biomarkers measure oxidative damage over different periods of time: 8-oxo-dG has a circulating half-life of hours[50] and protein carbonyls reflect exposure over a period of weeks.[51] Interestingly, we found no significant association between the two biomarkers themselves (Spearman $r = -0.006$; $P = 0.91$), adding support to previous reports of a lack of association between levels of oxidative damage to different types of biomolecules in humans.[52,53] Our data suggest that a single oxidative damage biomarker may be insufficient for drawing conclusions about oxidative damage in general. Finally, with 378 participants, this study was considerably larger than previous population-based studies of As exposure and oxidative damage.

There are also several potential limitations of this study. First, we measured oxidative damage in plasma and urine samples, but damage leading to As-related skin lesions and cancers may be tissue specific. It is possible that oxidative damage is occurring in tissues not sampled in this study. Nevertheless, previous positive reports of As-associated oxidative damage have also examined biomarkers in plasma[54,55] and urine,[35,56] as was done here. In addition, we note that peripheral blood mononuclear cells are a target for As toxicity,[57] so if chronic As toxicity to these cells occurs via oxidative damage, one might expect to observe evidence of it in plasma. Moreover, urinary 8-oxo-dG is viewed as a biomarker of "whole body" damage,[58] so if oxidative damage to DNA is significantly increased in other target tissues, one would expect this to be reflected in levels of this marker in urine.

The second limitation is that this study focused on whether drinking water As affected biomarkers of oxidative damage, but it has been shown that exposure to As can also occur through food items; this contribution is, relatively speaking, greater when water As levels are low.[59] In this study, data on dietary As exposure were not available. Nevertheless, we did measure As levels in both blood and urine, which reflect exposure from all sources, including the diet.

A third potential limitation is that the two markers of oxidative damage examined, plasma protein carbonyls and urinary 8-oxo-dG, were not associated with variables that might be expected to affect oxidative stress levels, such as current smoking status.[60–62] An association between smoking and these biomarkers has not been detected in many other studies, however.[63–68] In a low-income setting such as Araihazar, it is likely that many sources of oxidative damage, such as air pollution,[69,70] are present. Under these conditions, the level of damage associated with smoking may be relatively insignificant. Like us, other researchers have also failed to find an association between age and plasma protein carbonyls,[71,72] as well as urinary 8-oxo-dG.[73] Similarly, past studies have failed to find an association between sex and plasma protein carbonyls[74] and urinary 8-oxo-dG, after controlling for urine concentration.[75]

The mixed data from human population studies reported here and elsewhere contrast with the stronger and more-consistent evidence that As exposure results in oxidative damage obtained from in vitro[29,31] and animal experiments.[32–34] Although one important difference that may explain this discrepancy is the ready availability of target tissues in cell culture and animal experiments, it has also been shown that As exposure raises the levels of oxidative damage in blood[32] and urine[34] samples from rats. Thus, on the basis of laboratory experiments, one might expect that oxidative damage markers in blood and urine in human studies would also rise with As exposure. The doses of drinking water As used in animal experiments are often orders of magnitude higher and of shorter duration than those encountered by humans in natural settings.[29,32–34] It is possible that humans can better adapt to lower levels of As exposure over time and, to some extent, compensate for As-induced metabolic changes, thereby limiting oxidative damage.[76] In addition, species-specific differences in As metabolism and related health effects have been well documented,[77–79] and it is possible that such differences also apply to As-induced oxidative damage.

In summary, in this study, designed to detect dose–response effects of As on two biomarkers of oxidative damage in humans, we found no evidence that exposure to As increases levels of oxidative damage. No association was found between As and plasma protein carbonyl or 8-oxo-dG levels. A previous study on this sample has shown that As exposure is linked to a more-oxidized redox state, as measured by Eh values based on blood glutathione and glutathione disulfide.[38] It is possible that rather than manifesting in oxidative damage, As-induced changes in the redox state could influence the activity of redox-sensitive enzymes and thereby have downstream effects such as altered DNA methylation[80] or changes in gene expression.[81] Future studies should clarify the downstream effects of the more-oxidized cellular environment associated with As exposure.

ACKNOWLEDGMENTS

The authors thank Regina Santella and Qiao Wang for their help with the assays.

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